Für Papa, Sophie und Nicolas

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# An effect-based monitoring approach for environmental risk assessment of chemicals of emerging concern and complex chemical mixtures in the marine environment

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# Preface

In an attempt to explain how I ended up writing this PhD thesis, I think I have to go back in time (a little). Ever since our childhood, dad never ceased to repeat the tree's and animal's (especially bird's) names surrounding us in the garden or outside in general. To date, I have not encountered a person that showed more interest in the local flora and fauna. Whenever one project was finished (or often enough only half way), he came up with another idea on how to make our garden a little more environmental friendly. Piles of old wood or stones, insect hotels, various sorts of local fruit trees, just to name of few of his "innovations" to support animal welfare in our garden.

So while dad kept telling us how important it is to support local wildlife, after graduating from high school, I decided to explore some very distinct natural environment: VENEZUELA. 10.5 months abroad not only allowed me to learn the Spanish language but also incepted me with the idea that a life around the globe would be amazing. In an attempt to combine environment-related studies and globetrotting I started studying Bioscience Engineering (or agronomy as I used to call it) at AgroBioTech Gembloux in September 2007. Short story, I failed miserably! I won't try to explain the reasons why (French, laziness, wrong education system for my workwise) but rather look back at it as being a chance for a re-orientation. Obviously, it didn't result in a huge change since I was almost on my way to study agronomy in Bonn when the acceptance letter for studying biology in Aachen arrived. Great! Living at home, being able to continue playing soccer and scouting, and studying next door. The perfect combination and after finishing the Bachelor, "I can still go for a Master in an agronomy-related field". Might be that soccer, scouts and living at home contributed to the one year delay in finishing my Bachelor but I don't want to miss any of the time. Here a big shout-out to my fellow scout leaders and friends (Bagge "nowadays preferably "Raphaël", Blanc, Dave, Dome, Kaa (Dennis), Kaa (Tom), Maga, Maxime, Lena, PC, Ruth, Sebi, Titte, Zita,… Early on, in 2008, I further strengthened my bounds to Raeren by forming a couple with a local (scout) girl, Sophie. Living at home wasn't that bad after all!

Finishing our Bachelor's together in 2012 finally took me back to Venezuela for a whole month of traveling together with you. Great times and a moment to thank my host parents and siblings who considerably contributed to my personal development at a crucial time: mamá Gladys y Orangel con Angélica, Jhonángel y Mariángel, mamá Fabiola y papá Francisco con Mariana. Gracias por recibirme con tanto cariño y por estar en contacto hasta hoy en día! Ya no puedo esperar de volverlos a ver algún día.

But back to the educational track. Settled at home, and having delved into environmental sciences in the last year of my Bachelor's studies, I signed up for the relatively new Master course in ecotoxicology or "Ökotoxikologie" as the Germans call it. Öko…was? Yes indeed, it's a complicated term but at the end it (kind of) makes sense. A combination of environment (thanks dad!) and Toxicology (no clue how this fits into the family's history). Anyhow, the flexibility of the master's allowed me to re-discover the globetrotter that had been on hold for a few years. Excursions to Leipzig or the beautiful island Sardinia were only topped by 8 months in Stockholm, an unforgotten experience both socially and professionally. Together with starting the Master I was already speaking of the PhD that would follow and I still hear mom's and dad's voice: "Please take it one step at a time!" They seem to know me good enough to

forecast that it would again take longer than the foreseen 2 years to finish the Master. But, here I am, I guess! Keeping the promises I gave to myself back then.

Getting there would not have been possible without two people and I want to thank you, Karel and Colin for thinking sometime in June 2015 that I could be the right candidate to fill this PhD position. I certainly needed a while to pay back this trust and am grateful for giving me this opportunity. Colin, thanks for always being critical and straightforward but also sharing an encouraging advise every once in a while. Karel, if someone would ask me to describe the perfect supervisor for a PhD, I would shout your name! You perfectly managed the balance between giving me space and time to develop and try out things and close supervision to force advancements. Your input in our discussions offered so many possible tracks. After all those years, I'm still astonished when you start writing down some statistical formula from scratch! Thank you for carrying me through this PhD in the right moments and offering me the opportunity for personal development (e.g. via involvement in SETAC) in parallel. Finally, I also want to thank my colleagues. Thanks for heartily welcoming that German-speaking guy in Flanders to my favorite (West-)Flemings: Arne, Charlotte, Emmy, Gert, Gisèle, Ilias, Jana, Jolien, Jonathan, Karel (2), Karel (3), Olivier, Nancy, Niels, Sigrid, Wout and Yana. You made me feel welcome as of the beginning. Another shout-out to my international colleagues and friends: Abegail, Cecilia, Daniel, João, Natalia, Sharon, Simon, Tiptiwa, etc. I definitely felt like moving to a foreign country in the beginning, too! A few other people that also contributed considerably to the fact that Ghent felt like a second home were my housemates, Els (speciaal bedankt voor je bijdrage aan de voortdurende verbetering van mijn Nederlands), Tom and Mieke. Thank you all for the numerous activities and drinks we shared but also for advising (on my research) when needed and for any kind word in a tough phase. Of course, I would also like to thank the NewSTHEPS project colleagues, especially Camille, Francis, Steve, Kristof and Lynn. My fellow PhD students, Camille, Francis and Steve, thanks for unforgettable sampling campaigns and field trips but also fruitful discussions and collaborations. Finally, I would also like to thank the jury members for dedicating time to reading and commenting on my thesis. Your feedback helped to considerably improve this thesis and I enjoyed discussing my work with you.

Ook wil ik Marianne bedanken voor al je hulp en steun tijdens de voorbije vier en half jaren. Moest je in het begin nog zo goed als alle Nederlandstalige e-mails vertalen, kunnen we ondertussen gemakkelijk op Nederlands (of is het in het Gents?) praten. Dit ten minste wanneer je herinnert dat ik je nu ook in het Nederlands versta… Jou waarde voor het labo is onschatbaar en ik hoop dat we ten minste op de receptie van mijn verdediging nog een laatste foto voor onze EOTMD serie kunnen maken!

Uiteindelijk wil ik ook nog Emmanuel bedanken, je stekt uit als innig vriend. Thanks for the numerous adventures that enriched my weekly stay in Ghent. I hope to keep up with the good traditions and gather for future matches of the Red Devils in Brussels every now and then. Even though you failed at triggering my passion for cycling (that would probably have turned me into THE Belgian), I appreciate you for sharing and explaining every small detail of the Flemish culture. Also thanks for countless chats over lunch or after hours! You definitely heavily contributed to me enjoying every minute in Ghent.

Und dann ist da noch Josef. So lange wie unsere Wege sich jetzt schon kreuzen, bin ich fast schon versucht, schonmal einen Platz Bei P&G für dich zu reservieren! Danke für dein offenes

Ohr und deinen unschätzbar wertvollen Input. Dein Auge fürs Detail und deine so unterschiedliche Denkweise werden mir definitiv in Zukunft fehlen! Deine einfühlsame Weise und realistische Betrachtung der verschiedensten Situationen hat mir ein ums andere Mal den Kopf gerettet. Auch hätte ich mir keinen besseren Partner für die Organisation des YES Meetings vorstellen können. Danke für alles!

Die letzten viereinhalb Jahre waren vor allem durch Verzicht geprägt. Der Spagat zwischen Raeren und Gent war nicht immer selbstverständlich und wäre ohne euer mir entgegen gebrachtes Verständnis nicht möglich gewesen. Danke, dass ihr die Situation immer so akzeptiert und hingenommen habt, wie sie ist und trotzdem immer da wart, wenn ich euch brauchte, Nicolas und Zeno. Ihr seid und werdet immer eine zentrale Stütze in meinem Leben sein und ich freue mich auf die kommenden Jahre mit euch als meine besten Freunde. Danke auch an die Jungs von der Reserf'! Die regelmäßigen Siege sonntags morgens waren der perfekte Ausgleich zur meist geistigen Arbeit unter der Woche! Auf viele weitere Titel!

Die zweite zentrale Stütze ist ohne Frage meine Familie, Papa, Mama, Esther, Martin (mit Teresa und Henrik), Rebekka, Mätthi, Dagmar, Armin, Irina und Benno, Tobi und Jana, Anna, Oma. Was wurden wir gleich zu Beginn meines Doktorats gefordert. Den ersten Lichtblick 2016 gab es für uns mit Ausnahme des 20. Mai sicherlich erst am 20. September. Den Rest davor würden wir sicherlich alle am liebsten streichen. Papa, du fehlst! Und es sind eben solche Momente wie dieser, die das Verdrängen verdrängen. Ich muss ehrlich gestehen, dass ich mir die Zeit zum Erinnern viel zu selten nehme; dass ich kein Freund von Friedhöfen bin, ist glaube ich bekannt. Wie eingangs beschrieben, würde ich ohne dich sicherlich nicht hier sitzen und diese Zeilen schreiben. Es tut weh, Nicolas im Garten zu sehen und zu wissen, wie sehr du es genossen hättest, diesen mit ihm zu erkunden. Was uns bleibt ist die Erinnerung an viele wertvolle Momente mit dir und die Hoffnung, dass ich es auch nur ansatzweise schaffe, ein so guter Vater zu sein wie du. Und doch, hat 2016 auch positive Dinge hervor gebracht. Die Unterstützung durch viele gute Freunde und der Zusammenhalt den diese Zeit in unserer Familie gefördert hat, prägen uns auch heute noch. Ich denke, dass wir beispielsweise in dieser außergewöhnlichen Zeit davon profitieren. Für Mama, Esther und Rebbi sind Worte zu wenig. Danke für alles!

Liebe Sophie, diese vergangenen 4,5 Jahre haben schließlich auch dazu geführt, dass wir unsere eigene kleine Familie gegründet haben. Ohne deine Rückendeckung und die perfekte Ablenkung durch gemeinsames Spielen mit Nicolas wäre das alles sicherlich nicht möglich gewesen. Es ist schwierig die Dankbarkeit auszudrücken, die ich für deine Aufopferung in den vergangenen Jahren empfinde. Die wenigsten hätten wohl die Kraft und Bereitschaft, während fast 3 Jahren an fünf von sieben Tagen alleine ein Baby/Kleinkind groß zu ziehen. Danke! Nun freue ich mich, mit euch eine neue Etappe in unserem gemeinsamen Leben anzugehen und bin gespannt, was die Zukunft für uns bereit hält. Ich kann euch lediglich versprechen, jederzeit für euch da zu sein, so wie ihr es in den vergangen Jahren wart. Sophie, ich liebe dich und bin dankbar für jeden gemeinsamen Moment. Nicolas, ich genieße jede Sekunde mit dir und bin unendlich dankbar tagtäglich an deiner Seite zu Wachsen. Und weil die Antwort auf *„Wo ist Papa?"* in den vergangenen 3 Jahren vermutlich viel zu oft *„Bei der Arbeit"*, *„In Gent"* oder *„Im Büro"* war, sollst du zumindest auch einen kleinen Beitrag zu dieser Arbeit beisteuern dürfen: *"333333333333333222222111456987/*"*.

Raeren, June 2020]

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## Summary

Along with a worldwide growth of the human population, the aquatic environment on our planet is facing an ever increasing chemical input. With the aim of regulating chemical use and protecting both humans and the environment a number of regulatory frameworks for chemicals have been introduced in the European Union (EU). Yet, these regulations mainly focus on a limited list of priority pollutants that represent only a minor fraction of potentially water-emitted chemicals. In addition, even though there exist regulatory frameworks for the marine environment, risk assessment for the latter does not require ecotoxicity data for marine species. To partly address the herewith associated ecotoxicity data gap for marine species, in the present work, marine ecotoxicity data was generated for a total of 23 chemicals of emerging concern (CECs) with two species representing algae and crustacean (Chapter 4). While algae did show low sensitivity to all tested substances, relatively low effect concentrations on crustacean were found for 4 neonicotinoid insecticides. Acute and sub-chronic ecotoxicity data for crustacean was subsequently used together with existing literature data to derive Environmental Quality Standards (EQS) for the marine environment. Inclusion of the marine copepod data from this study led to a refinement of the EQS for clothianidin and thiamethoxam. An in-depth risk assessment for the Belgian part of the North Sea (BPNS) based on the derived EQS for the 4 neonicotinoid insecticides and their mixture (Chapter 4) resulted in an exceedance of predicted no-effect concentrations (PNECs) in the harbors of Ostend and Zeebrugge and a low margin of safety (MoS) for one coastal locations in front of each of these harbors.

Such derivation of EQS and risk assessments are time-intensive processes that require in-depth ecotoxicological and regulatory knowledge. Considering the huge amount of chemicals present in the marine environment, from a regulatory perspective, there are only two promising approaches to handle this complex task: i) automation of EQS/PNEC derivation and associated risk assessment on a substance-by-substance level or ii) moving from a single substance-based to a mixture-based risk assessment. Therefore, in Chapter 5 an automated calculation algorithm was developed and applied in a screening-level risk assessment for the BPNS. This screening-level marine risk assessment suggests to prioritize in future work Bisphenol A, certain herbicides, neonicotinoid insecticides and steroids for further ecotoxicological testing and/or refined PNEC calculation. Additionally, a comparison of grab sample and passive sampler-based risk assessment revealed no obvious differences between the two sampling methods.

Although providing a useful tool for prioritization within the prevailing regulatory frameworks, a single-substance-based risk assessment bears the risk of neglecting interactive effects of chemicals. Yet, environmental risk assessment is meant to assess the real impact on ecosystems or species that are exposed to chemicals and for most of our waters this means simultaneous exposure to various chemicals. This indicates that there is a need for mixture-based risk assessment methods. To answer this need, we developed a novel method for passive sampler-based ecotoxicity testing of environmentally realistic chemical mixtures (ERCMs) in Chapter 6. This passive sampler-based method combines environmental sampling and ecotoxicity testing of chemical mixtures. During method development insights into the preservation of complex mixture samples were gained and the importance of a reduction of

passive sampler extract handling and storage time were highlighted. With a relatively low sample enrichment of < 2 the developed method had one major drawback.

This drawback was tackled in Chapter 7 where the previously developed method was modified to allow sample enrichment up to a relative enrichment factor (REF) of 44 as compared to environmentally realistic concentration levels. Further, the method was extended with a MoS assessment serving as indicator for potential risks in the BPNS. Here, margins of safety were found to be < 10 for 5 out of 8 samples from different sampling campaigns (SCs) and locations. According to current risk assessment procedures this suggests ecological risks for these locations since the lowest assessment factor (AF) in use is 10. This effect-based method addresses the lack of current environmental regulations that do not provide guidance on how to deal with mixtures of chemicals although simultaneous exposure to multiple chemicals is the prevailing scenario for aquatic organisms. Yet, a change in environmental regulation from single substance to mixture-based risk assessment is not to be expected in the near future since many of the current EU frameworks have only recently entered into force. Thus, to align our effect-based monitoring method with current risk assessment procedures, we recommend to extend the biotest battery with at least one crustacean and one fish biotest to comply with regulatory requirements.

In a first attempt to identify mixture toxicity driving chemicals, we applied multivariate statistics to find chemical concentration patterns in different speedisk extracts that might be associated with the toxicity observations (Chapter 7). Unfortunately, no clear patterns distinguishing between toxic and non-toxic samples could be identified based on 89 target personal care products (PCPs), pesticides and pharmaceuticals. Nevertheless, some chemicals like sodium diclofenac or naproxen, that had also been identified in the screening-level risk assessment to be potentially problematic substances, were found to be correlated with the first two principal components (PCs) that explained 55 % of the data inherent variability. Yet, in order to gain better insights into the effect-driving chemicals in a mixture including non-target chemical data is highly recommended. This would be another step forward from a single substance-based and priority pollutant-focused to an unbiased mixture-based risk assessment.

# Samenvatting

Gepaard met de toename van de menselijke bevolking worden de aquatische milieus op onze planeet geconfronteerd met een toenemende antropogene input van chemicaliën. Om het gebruik van chemicaliën te reguleren en zo mens en milieu te beschermen, werden in de Europese Unie (EU) een aantal regelgevingskaders geïntroduceerd. Toch focussen deze regelgevingskaders zich voornamelijk op een gelimiteerde lijst van prioritaire polluenten die slechts een beperkt aandeel van de chemicaliën, die in het aquatische milieu terecht komen, vertegenwoordigen. Ondanks de verschillende bestaande wetgevende kaders voor het marine milieu, is er bovendien geen ecotoxicologische data verreist van mariene species om een risico analyse voor het marine milieu uit te voeren. Het voorgestelde werk wijst op dit gebrek van realisme en ondervertegenwoordiging van mariene species in milieurisico-evaluatie voor de zee. In een eerste fase van dit werk werd er ecotoxicologische data voor 23 nieuw-opkomende polluenten, voor twee verschillende marine soorten (één alg en één soort roeipootkreeftje), gegenereerd (hoofdstuk 4). Terwijl het testen van algen een eerder lage sensitiviteit aantoonde voor alle geteste stoffen, werden er voor 4 neonicotinoïden relatief lage effect concentraties voor het roeipootkreeftje gedetecteerd. De hieruit volgende acute en sub-chronische data werden vervolgens, samen met data uit de literatuur en actieve en passieve monstername in de Noordzee, gebruikt om Ecologische Kwaliteit Standaarden (EQS) te bepalen voor het marine milieu. Deze nieuw-gegenereerde ecotoxiciteit data voor marine roeipootkreeftjes resulteerden in een aanpassing van de EQS voor clothianidin en thiamethoxam. Een gedetailleerde milieurisico-evaluatie voor het Belgisch deel van de Noordzee (BPNS), gebaseerd op de afgeleide EQS voor de 4 neonicotinoïden en hun mengsel (hoofdstuk 4), resulteerde in een overschrijding van de voorspelde geen-effect concentratie (PNEC) in de havens van Oostende en Zeebrugge en een beperkte veiligheidsmarge (MoS) voor de open zeegebieden dichtbij deze havens.

Het afleiden van EQS en milieurisico-evaluatie en zijn tijdrovende processen waarvoor een gedetailleerde ecotoxicologische en wetgevende kennis verreist is. Gezien het feit dat er een enorm aantal chemicaliën in het marine milieu terecht komen, zijn er uit vanuit een regelgevend perspectief slechts twee veelbelovende pistes voor deze complexe uitdaging: i) de automatisatie van de EQS/PNEC afleiding en de geassocieerde milieurisico-evaluatie op een stof per stof basis of ii) de omslag van een enkele stoffen-gebaseerde naar een mengsel-gebaseerde aanpak voor milieurisico-evaluaties. In hoofdstuk 5 werd bijgevolg een geautomatiseerd berekenings-algoritme ontwikkeld en toegepast voor een screening-level milieurisico evaluatie van het BPNS. Deze screening-level milieurisico evaluatie voor het marine milieu verreist toont aan dat er verder onderzoek verreist is voor Bisphenol A en enkele herbiciden, neonicotinoïden, insecticiden en steroïden. Verder werden er geen duidelijke verschillen waargenomen tussen de milieurisico evaluaties die gebaseerd waren op actieve en passieve monstername methodes.

Ondanks dat een geautomatiseerde benadering handig is voor een screening-level milieurisico-evaluatie en het prioriteren van chemische stoffen, bestaat het risico dat interactie effecten van chemicaliën worden genegeerd. Gezien een milieurisico-evaluatie de reële invloed van chemicaliën op organismen en ecosystemen dient te bepalen, betekent dit voor de meeste wateren de invloed van een simultane blootstelling aan een hele mix van chemicaliën. Er is er dus nood aan mengsel-gebaseerde methoden voor milieurisico-evaluatie.

Om hieraan te voldoen werd er in hoofdstuk 6 een methode ontwikkelt voor het ecotoxicologisch testen van milieu relevante en aangereikte mengsels op basis van passieve monstername technieken. Deze holistische methode implementeert milieustalen in ecotoxicologische testen en analyseert zo heel realistische mengsels van chemicaliën. De ontwikkeling van deze methode gaf verder ook inzichten over de conservering van deze stalen met complexe mengsels. Bovendien werd het belang van een beperkte verwerking van "passief-sampler" extracten en hun bewaring beklemtoond. In het algemeen leverde de ontwikkelde methode slechts een beperkte aanrijking van de stalen op (relatieve aanrijkingfactor, REF < 2), waardoor er aanpassingen verreist waren.

Deze aanpassingen werden in hoofdstuk 7 uitgevoerd, waar de ontwikkelde methode werd aangepast. Met deze aangepaste methode waren veel hogere (< 44) REFs mogelijk, in vergelijking met de milieuconcentraties van de bemonsterde chemicaliën. Deze methode werd tevens uitgebreid met de berekening van een MoS inschatting voor het BPNS. Op deze manier werden MoS < 10 gevonden voor 5 van 8 stalen. Volgens huidige milieurisico-evaluatie procedures suggereren deze resultaten een ecologisch risico aangezien dat de laagst gebruikte beoordelingsfactor (AF) 10 is. Deze effecten-gebaseerde methode duidt de tekortkomingen van het huidig wetgevende kader aan, die geen instructies voor mengsels van chemicaliën omvat. Toch is er in de nabije toekomst geen verandering van een enkele stoffen gebaseerde milieurisico-evaluatie na een mengsel gebaseerde milieurisico-evaluatie in de EU te verwachten, gezien het merendeel van de wetgevende kaders inzake milieu nog maar enkele jaren in werking zijn. Om onze effecten gebaseerde monitoringsmethode aan het huidige wetgevende kader aan te passen, raden we bijgevolg aan om de biotest-batterij uit te breiden met minstens één test voor schaaldieren en één test met vissen.

In een eerste poging om de effect-veroorzakende stoffen in mengsels te bepalen, werd een multivariate statistiek gebruikt om bepaalde trends in de chemische compositie van verschillende extracten te vinden en de toxische effecten zo te kunnen verklaren (hoofdstuk 7). Helaas werden geen trends gevonden om toxische en niet toxische monsters te onderscheiden op basis van gerichte chemische analyses voor 89 stoffen. Deze 89 stoffen omvatten waaronder schoonmaak- en lichaamsverzorgingsmiddelen (PCPs), pesticiden en farmaceutica. Desondanks konden sommige chemicaliën zoals diclofenac natrium en naproxen, die ook in de screening-level milieubeoordeling als potentieel problematisch werden geïdentificeerd, met de twee belangrijkste componenten (PCs) worden gecorreleerd. Deze twee PCs verklaarden 55 % van de inherente data variabiliteit. Om verdere inzichten over de effect-veroorzakende stoffen in mengsels te bekomen, werd aangeraden om ook *untargeted* chemicaliën te analyseren. Dit zou een belangrijke stap zijn om van een enkele stoffen en prioritaire stoffen gebaseerde naar een objectieve mengsel gebaseerde milieurisico-evaluatie te gaan.

## List of abbreviations

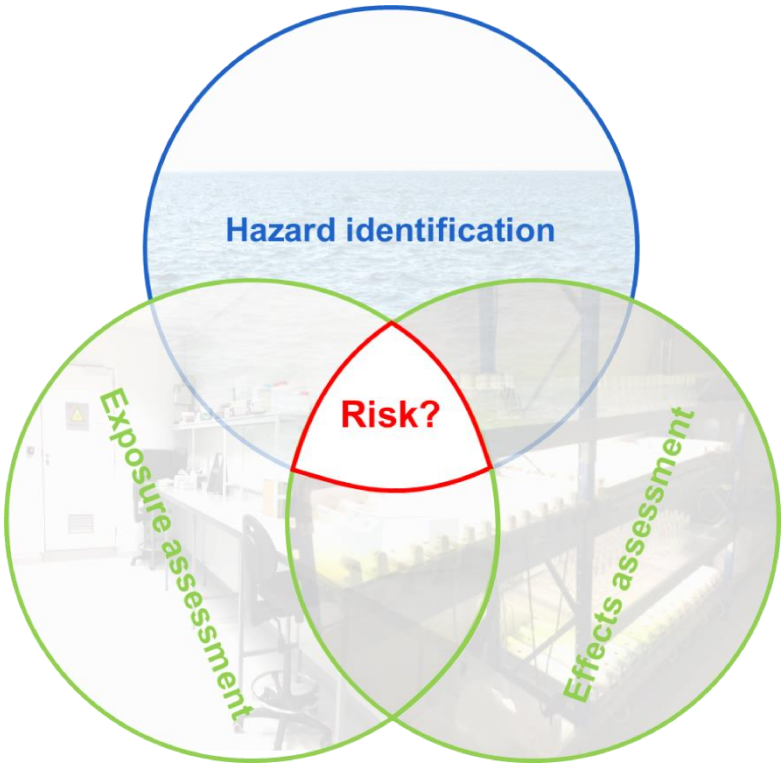
| | | |
|---|---|---|
| **A** | | |
| | AA | Annual Average |
| | AF | Assessment Factor |
| | AI | Active Ingredient |
| | ANOVA | Analysis of Variance |
| **B** | | |
| | BPNS | Belgian Part of the North Sea |
| | BPR | Biocidal Products Regulation |
| **C** | | |
| | CA | Concentration Addition |
| | CALUX | Chemical Activated Luciferase Gene Expression |
| | CCAP | Culture Collection of Algae and Protozoa |
| | CEC | Chemicals of Emerging Concern |
| | CI | Confidence Interval |
| | CL | Confidence Limit |
| | CLO | Clothianidin |
| | CT | Concentration Treatment |
| | CV ANOVA | Cross-Validated Residuals Analysis of Variance |
| **D** | | |
| | DEET | Diethyl-toluamide |
| | DEHP | Diethyl-hexyl-phthalate |
| **E** | | |
| | E1 | Estrone |
| | E2 | 17β-Estradiol |
| | $EC_x$ | Effect concentration affecting x % of the Test Organisms |
| | EDA | Effect-Directed Analysis |
| | EE2 | 17α-Ethinylestradiol |
| | EFSA | European Food Safety Authority |
| | EGD | European Green Deal |
| | EPA | Environmental Protection Agency |
| | EQS | Environmental Quality Standards |
| | ERCM | Environmentally Realistic Chemical Mixture |
| | EU | European Union |
| **G** | | |
| | GES | Good Environmental Status |
| | GF/D | Glass Fiber Type D |
| **H** | | |
| | HC5 | Hazardous Concentration affecting 5 % of the Species |
| | HLB | Hydrophilic-Lipophilic Balance |
| | HO | Harbor of Ostend |
| | (U)HPLC | (Ultra-)High Performance Liquid Chromatography |
| | HRMS | High-Resolution Mass Spectrometry |
| | HZ | Harbor of Zeebrugge |
| **I** | | |
| | IA | Independent Action |

| | | |
|---|---|---|
| | IMI | Imidacloprid |
| | ISO | International Organization for Standardization |
| **K** | | |
| | $K_{OW}$ | Octanol-Water Partitioning Coefficient |
| **L** | | |
| | LB | Life Benchmark |
| | $LC_x$ | Lethal concentration affecting x % of the Test Organisms |
| | LDR | Larval Development Ratio |
| | LL | Log-Logistic |
| | LOC | Level of Concern |
| | LOEC | Lowest-Observed Effect Concentration |
| | LV-SPE | Large Volume-Solid Phase Extraction |
| **M** | | |
| | MAC | Maximum Allowable Concentration |
| | MCA | Multi-Criteria Assessment |
| | MDL | Method Detection Limit |
| | MEC | Measured Environmental Concentration |
| | MoS | Margin of Safety |
| | MS | Mass Spectrometry |
| | MSFD | Marine Strategy Framework Directive |
| **N** | | |
| | NewSTHEPS | New Strategies for Monitoring and Risk Assessment of Hazardous Chemicals in the Marine Environment with Passive Samplers |
| | NOAEC | No-Observed Adverse Effect Concentration |
| | NOEC | No-Observed Effect Concentration |
| | NOEL | No-Observed Effect Level |
| **O** | | |
| | OECD | Organization for Economic Cooperation and Development |
| | OO_X | Coastal sampling location near Ostend |
| | OPLS-DA | Orthogonal Partial Least Squares Project to Latent Structure-Discriminant Analysis |
| | OPP | Office of Pesticide Programs |
| | OZ_MOW1 | Coastal sampling location near Zeebrugge |
| **P** | | |
| | PBT | Persistent, Bio-accumulative, Toxic |
| | PC | Principal Component |
| | PCA | Principal Component Analysis |
| | PCP | Personal Care Product |
| | PDMS | Polydimethylsiloxane |
| | PEC | Predicted Environmental Concentration |
| | PNEC | Predicted No-Effect Concentration |
| | POCIS | Polar Organic Chemical Integrated Sampler |
| **R** | | |
| | RCR | Risk Characterization Ratio |
| | REACH | Registration, Evaluation, Authorization and Restriction of Chemicals |
| | REF | Relative Enrichment Factor |

| | | |
|---|---|---|
| | RPS | Reverse Phase Sulfonate |
| | RQ | Risk Quotient |
| **S** | | |
| | SC | Sampling Campaign |
| | SDB | Styrene-divinylbenzene |
| | SPE | Solid Phase Extraction |
| | SPMD | Semi-Permeable Membrane Devices |
| | SR | Silicone Rubber |
| | SSD | Species Sensitivity Distribution |
| **T** | | |
| | TCP | Thiacloprid |
| | TGD | Technical Guidance Document |
| | TMX | Thiamethoxam |
| | TTC | Threshold of Toxicological Concern |
| | TU | Toxic Unit |
| **U** | | |
| | US | United States |
| **V** | | |
| | VIP | Variable Importance in the Projection |
| **W** | | |
| | WFD | Water Framework Directive |

# 1

# General introduction and conceptual framework



# 1 General introduction

## 1.1 Legislative background

The Marine Strategy Framework Directive (MSFD) entered into force in June 2008 and aims to achieve or maintain good environmental status (GES) of the marine environment by the year 2020 [1]. GES of marine waters is determined by a total of 11 descriptors of which descriptor 8 asks that “concentrations of contaminants are at levels not giving rise to pollution effects” [1]. Next to the MSFD, the Water Framework Directive (WFD), which entered into force in October 2000, describes a strategy to fight pollution of water by progressive reduction or phasing-out of discharges and emissions [2]. In support of the aims of the WFD, the EQS Directive establishes requirements for the chemical status of surface waters, including marine waters, by setting EQS [3]. Under the latest amended version of the directive, EQS have been defined for 45 substances or substance groups [4]. This list contains priority substances [5] as defined under the WFD and defines EQS for surface and marine waters. Remarkably, EQS derivation for marine waters does not strictly require any ecotoxicological data for marine test species [6]. This might be one reason as to why the availability of marine ecotoxicity data is certainly less than optimal for marine environmental risk assessment [7].

Along with a worldwide growth of the human population, the aquatic environment on our planet is facing an ever increasing chemical input [8]. With the aim of regulating chemical use and protecting both humans and the environment a number of regulatory frameworks for chemicals have been introduced in the EU. These regulations target different substance groups depending on their use and industry. Major EU regulations include the Biocidal products regulation (BPR) [9], the EU Pesticides regulation [10], human and veterinary medicines regulations [11] and the regulation concerning the registration, evaluation, authorization and restriction of chemicals (REACH) [12].

## 1.2 Chemicals in the environment

With the third and last REACH deadline having passed on 31st of May 2018, 10,119 substances with a production volume above one ton per year have been registered in the EU. In addition, 10,489 substances with a production volume below one ton per year or with intermediate use only have been registered [13]. In the decade 2000 to 2010, the American Chemistry Council calculated a total chemical production increase of 54 % which reflects the general trend in chemical production worldwide [14] and global chemicals sales have been predicted to grow about 3 % per year until 2050 [14]. Along with an increasing production and consumption more chemicals are expected to be released into the aquatic environment and ultimately into marine waters [8]. The major input routes for chemicals into the aquatic environment are depicted in ***Figure 1.1***. Several man-made chemicals are very likely to be released into the aquatic environment and might ultimately be transported into marine waters. Evidence for marine waters being a kind of repository for chemicals is given by various recent chemical monitoring publications. For instance, a variety of chemicals have been detected in European marine waters ranging from the Aegean [15] and Adriatic Sea [15], over the Mediterranean Sea [15, 16] to the North Sea [17-20] and the Baltic Sea [15].

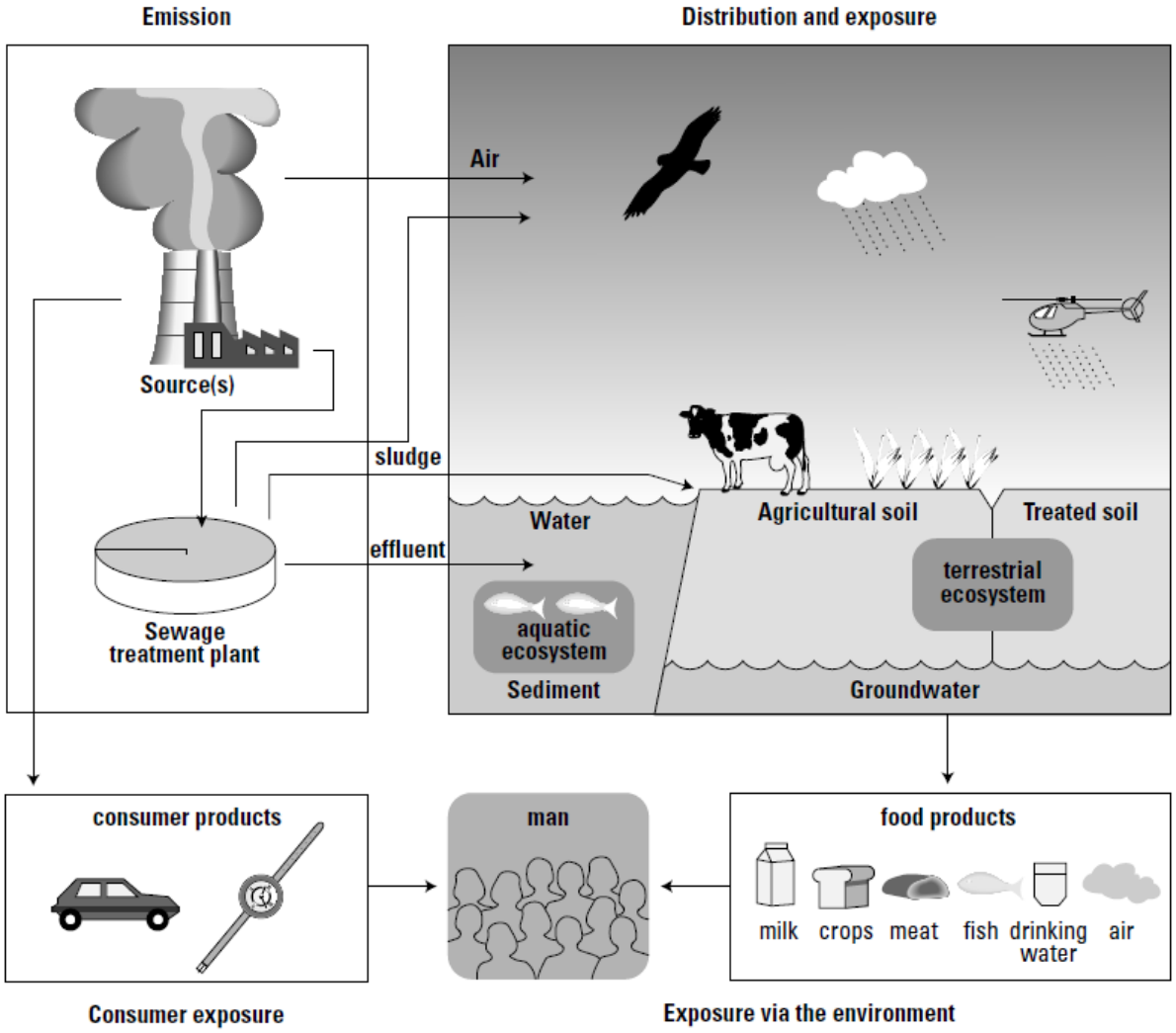



**Figure 1.1 Major input and distribution routes of man-made chemicals in the environment [21].**

## 1.3 Environmental chemical monitoring

Environmental monitoring in the EU is mostly focused on a limited set of priority pollutants as defined by the European Commission [22]. Nevertheless, it is recognized that not all substances on the priority list are still representative of present day contamination [23] and other, non-monitored substances are becoming of emerging concern. Most aquatic monitoring programs rely on the analysis of water samples taken at one location at a specific time, i.e. grab sampling [24]. Grab sampling is a fast approach that requires moderate sample clean-up and processing before analysis but is generally limited to a point measure [25]. This may be problematic for streams with highly variable chemical concentrations but has less influence on generally well equilibrated marine waters. Nevertheless, in order to detect chemicals at rather low concentrations such as often occurring in marine waters, high sample volumes are needed. This is associated with increasing extraction duration that can substantially compromise the integrity of the original sample [25]. This is a considerable disadvantage when coupling monitoring with ecotoxicological studies to assess potential effects of natural chemical mixtures. Another, but less frequently used approach is biomonitoring. Here, live organisms are either sampled from or deployed in a study area over a prolonged time where they “sample”

chemicals continuously. Typical organisms used in this form of monitoring include (caged) bivalves (e.g. oysters) [26, 27] and (caged) fish (e.g. trout) [28, 29]. Biomonitoring delivers a time-integrative picture of chemical concentration levels but has a set of limitations. Live organisms are limited in where they can be deployed due to a variety of surrounding factors essential to their survival such as e.g. salinity, temperature or water quality [25]. In addition, differences in age or sex have influence on chemical uptake rates of organisms of the same species [30]. A third aquatic monitoring method is passive sampling. Here, a sorption phase is exposed to a medium (e.g. water), where it samples compounds at a rate that is proportional to the difference in chemical activity between sampler and medium, and where the uptake kinetics are controlled by passive processes, until equilibrium is reached [31]. Passive sampling devices are usually divided into two categories either aiming at the uptake of polar or non-polar chemicals [32]. An overview of the multitude of available passive sampling devices and their intrinsic properties is given in Vrana et al. (2005) [24].

## 1.4 Environmental risk assessment

Environmental risk assessment is based on three major pillars, hazard identification, exposure assessment and effect assessment.. According to the European Commission's Technical guidance document (TGD) on risk assessment, an environmental risk assessment should be carried out on notified substances, substances of concern in a biocidal product and on priority existing substances and active substances [33]. Further, it should proceed in the sequence i) hazard identification, ii) dose (concentration) – response (effect) assessment, iii) exposure assessment and iv) risk characterization [33]. This risk characterization is ultimately expressed as a risk characterization ratio (RCR) or risk quotient (RQ) calculated as the ratio of the measured or predicted environmental concentration (MEC/PEC) and the predicted no-effect concentration (PNEC), where a RCR > 1 indicates a risk ***(Eq. 1.1)***.

$$RCR \text{ or } RQ = \frac{MEC \text{ or } PEC}{PNEC} \qquad \textbf{(Eq. 1.1)}$$

### *1.4.1 Hazard identification*

The aim of a hazard identification is to identify chemicals of concern and their intrinsic effects of concern [33]. The overall aim for known substances and biocidal products is to review the classification of the substances [33]. For new (emerging) substances the aim is a proposal on classification [33]. A hazard describes a potential source that may or may not cause harm while a risk includes the likelihood for a certain hazard to cause harm. As such, the hazard of a substance can be very high but if the likelihood of exposure to this substance is minuscule, the overall risk is low. Hazard identification is traditionally based on data of known compounds that may either be generated for a specific compound of interest or can (in some cases) be

predicted using e.g. QSARs based on structurally similar compounds. In order to adequately assess potential risks of a chemical both effects and an exposure assessment are required.

### *1.4.2 Effect assessment*

In order to assess whether the presence of a substance is harmful to an ecosystem, information on its toxic potential is needed. This information is generally obtained on a substance-by-substance basis through laboratory ecotoxicity testing under controlled conditions or via read-across from similar chemicals. For the sake of comparability such testing is ideally performed according to existing test guidelines such as from the International Organization for Standardization (ISO). An example for one of the most common ecotoxicity tests is the fresh water algal growth inhibition test with unicellular green algae [34] in which algae are exposed to several concentrations of a test substance for 72h and algal growth is monitored over time. Such ecotoxicity tests result in the definition of effect thresholds such as the effective or lethal concentrations affecting 50 % of the test organisms ($EC_{50}$ or $LC_{50}$) for acute tests or effect concentrations affecting 10 % of the test organisms ($EC_{10}$) and no observed effect concentrations (NOEC) for chronic tests. The $EC_{50}$ or $LC_{50}$ and the $EC_{10}$ are statistically derived by fitting an appropriate model to the observations. One of the most commonly used models in ecotoxicology is the dose/concentration-response model. Here, the effect is plotted over a (log-transformed) concentration range and a sigmoidal curve is fitted to allow determination of the concentration that results in e.g. 50 % effect. An example for a dose-response curve is given in ***Figure 1.2***.

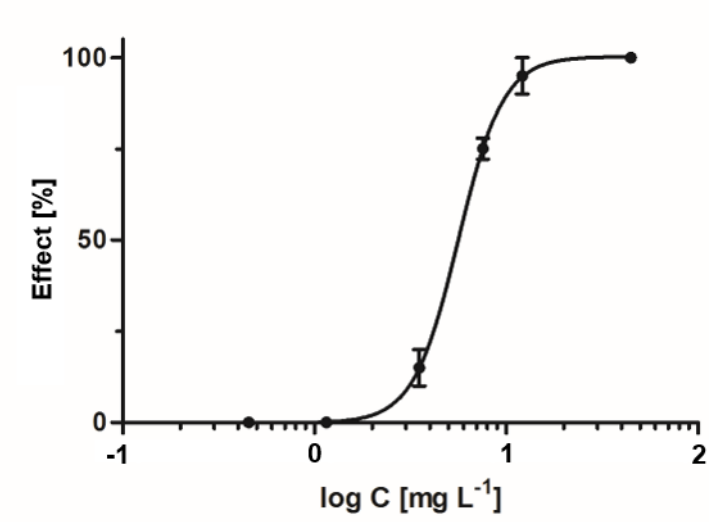



**Figure 1.2 Illustration of a concentration-response curve showing data points as average of replicates including their standard deviation. The solid line shows a typical sigmoidal concentration-response curve fitted with a log-logistic model. Such a model allows the estimation of e.g. $EC_{50}$ values, the concentration that exerts an effect to 50 % of the test organisms.**

In addition, there exists a statistical extrapolation method called species sensitivity distribution (SSD). Here, usually the 5$^{th}$ percentile, often referred to as HC5 is derived from a distribution function fitted on log-transformed ecotoxicity data (usually $EC_{10}$ or NOEC values), preferably from at least 10 species representing 8 different taxonomic groups [6]. The lowest effect concentration (acute, chronic endpoint or HC5) is subsequently divided by an AF to calculate

a PNEC. AFs are used to account for uncertainties associated with the extrapolation from single-species laboratory ecotoxicity data to a multi-species ecosystem [33]. These uncertainties include i) intra- and inter-laboratory variation of toxicity data, ii) intra- and inter-species variation (biological variance), iii) short-term to long-term toxicity extrapolation or iv) laboratory data to field impact extrapolation [33]. The size of an AF depends on the confidence in the amount, type and quality of data a PNEC derivation is based on. Depending on the target ecosystem (freshwater or marine) and the data availability, AFs vary between 5 for SSD-derived HC5 values and 10 – 10,000 for single species-derived endpoints [33] and are applied to the lowest effect concentration or HC5 to obtain the PNEC ***(Eq. 1.2)***.

$$PNEC = \frac{Lowest\ effect\ concentration\ or\ HC5}{AF} \qquad \textbf{(Eq. 1.2)}$$

### *1.4.3 Exposure assessment*

For the exposure assessment information about emission rates, physico-chemical properties and fate and behavior of a substance, and environmental conditions are required. Such assessment can be retrospective by sampling at a specific area or location of interest (as described in Chapter 1.3) or predictive by extrapolating from use, distribution and fate of (a) chemical(s). The latter assessment is usually applied to predict the exposure concentrations for novel substances and is mostly based on default scenarios [35] to calculate a PEC. Chemicals in the environment always migrate between the different compartments driven by equilibrium partitioning. The marine environment for example consists of four compartments, i.e. air, water, sediment and biota. For the majority of organic chemicals the distribution across these compartments is defined by their intrinsic physico-chemical properties. As an example, a non-polar substance with a high octanol-water partitioning coefficient (log $K_{OW}$), i.e. low water solubility, and high organic carbon-water partitioning coefficient (log $K_{OC}$), will tend to accumulate in biological tissues (biota) and sediments. In contrast, a polar substance is more likely to be found at comparably high concentrations in the water phase as compared to e.g. sediment. From an ecotoxicological point of view, chemicals with the greatest concern are those with persistent, bioaccumulative and toxic (PBT) properties. The distribution of a chemical across the different compartments can be estimated using distribution models. Exposure modelling within the NewSTHEPS project for the neonicotinoid insecticide clothianidin in the BPNS predicted the concentrations to fluctuate approximately 10-fold at OZ_MOW1 (0.11 – 1.1 ng $L^{-1}$). Additionally, input modelling for the three rivers Meuse, Rhine and Scheldt identified a considerable fluctuation of the simulated relative contribution of clothianidin in the BPNS with each of the rivers showing a period in which they contribute the most. Ultimately, a clear influence of the degradation half-life on the relative contributions was found with slower degradation rates (e.g. 100 d) resulting in higher clothianidin concentrations at OZ_MOW1.

### *1.4.4 Risk characterization*

The risk characterization combines the information gathered from both effect and exposure assessment to calculate a RQ (Equation 1.1). This is traditionally done on a substance-by-substance level [36, 37]. However, various environmental monitoring campaigns have revealed the simultaneous presence of a variety of substances [15-18, 20] in marine waters.

Based on these observations, awareness for the necessity of characterizing the risk(s) of chemical mixtures is receiving increased recognition [38].

## 1.5 Mixture toxicity

Aquatic organisms are usually exposed to mixtures of chemically and functionally heterogeneous compounds [39-43]. Single substance-based risk assessment might thus lead to an underestimation of the total risk [42, 44] and the question arises whether reliable predictions of aquatic toxicity of chemical mixtures can be derived from toxicity data obtained from single substance tests. This is increasingly problematic since various studies have reported effects of chemical mixtures at concentrations of substances that individually did not cause any effects [40, 45-49].

Therefore, concepts and models for predicting mixture toxicity are needed. One approach is to base the prediction of the overall mixture toxicity on the knowledge about the hazard of individual substances. This implies that the chemical composition of the mixture of interest is known [46]. In practice, this is usually not the case but currently two major concepts are used to predict mixture toxicity on the basis of the mixture's components. The two concepts are concentration addition (CA) [50] and independent action (IA) [51]. Both concepts in theory only consider cases in which all components of the respective mixture do affect the same endpoint and both require precise knowledge about the qualitative and quantitative composition of the mixture of interest [46]. CA is used to predict the mixture toxicity of substances with a common target site and a similar mode of action. It assumes that the components of a mixture differ only with respect to their individual potency [52]. Any component of the mixture can be replaced by an equal fraction of an equally effective concentration of another chemical without changing the overall toxicity as long as the corresponding toxic units (TU) are identical. The TU approach implies that the relative toxicological strength of every mixture component may be expressed by scaling the individual concentrations of the single components in the mixture for their respective toxicity. Here, the $TU_i$ is the TU for mixture component i, or the ratio between measured concentration of each component i ($c_i$) and its $EC_{x,i}$ and n indicates the number of mixture components considered ***(Eq. 1.3)***.

$$\sum_{i=1}^{n} TU_{i,x} = \sum_{i=1}^{n} \frac{C_i}{EC_{x,i}} \qquad \textbf{(Eq. 1.3)}$$

The CA concept implies that substances present at a concentration below their individual no observed effect concentration (NOEC) can nevertheless contribute to the total effect of the mixture [41]. The second concept, IA, is used to predict the hazard of mixtures of chemicals with dissimilar mechanisms of action and different target sites [40]. The alternative concept of IA can be calculated using **(Eq. 1.4)**.

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad \textbf{(Eq. 1.4)}$$

Here, $E(c_i)$ describes the effects of substance i and $E(c_{mix})$ the total effect of the mixture. Contrary to CA, under IA mixture components present below their individual NOEC do not contribute to the mixture effect, i.e. there will be no mixture effect if all substances are present below their NOEC [40].

Even though both concepts are largely used and discussed, there are still some critical points regarding the prediction of mixture toxicity. For both concepts the similarity or dissimilarity of the modes of action of the mixture components is the governing factor for the prediction quality of the concepts. If the corresponding mechanistic assumptions are fully met, both concepts give good predictions, but CA tends to overestimate the mixture toxicity of dissimilarly acting chemicals and IA tends to underestimate the toxicity of similarly acting substances [53]. One main problem is the interpretation of the term of similar modes of action. From a strict mechanistic point of view it should be applicable only to such chemicals that competitively and reversibly interact with an identical molecular binding site. But from a very broad phenomenological point of view it may just describe different chemicals that are able to cause a common toxicological endpoint such as death, inhibition of reproduction. This might apply to almost all chemicals [45, 54]. It is the rule rather than the exception that agreement about similarity or dissimilarity of action cannot be reached. Additionally, knowledge about mechanisms changes and expectations about presumed modes of action do not necessarily match biological observation. But, lack of knowledge about modes of action should not prevent the use of both concepts [55].

Both concepts can also be used for risk assessment purposes and especially the TU approach has been used by replacing the effect concentration in ***(Eq. 1.3)*** by the substance specific PNEC ($PNEC_i$) for each of the mixture components as follows:

$$RQ_{mix} = \sum_{i=1}^{n} TU_{i,x} = \sum_{i=1}^{n} \frac{C_i}{PNEC_i} \qquad \textbf{(Eq. 1.5)}$$

Ultimately, application of both concepts depend on the knowledge of the mixture composition which in environmental practice is usually not available. Thus, for the purpose of risk characterization of complex mixtures novel effect-based monitoring approaches are needed [38].

## 1.6 Rationale for this thesis

This study was conducted as part of the NewSTHEPS project (www.newstheps.be). This project aimed to develop innovative and novel practical techniques to address the current fundamental scientific and methodological issues related to the evaluation of GES for Descriptor 8 of the MSFD in national and European waters. This included the development, application and testing of novel and integrated passive sampler-based approaches for both chemical exposure (monitoring) and biological effect assessment. Further, it aimed to assess the ecotoxicity of both CECs and ERCMs and to develop alternative approaches for a mixture-based risk assessment in the marine environment.

In an assessment report about the proposed road map to comply with the MSFD obligations (published in 2014) Belgium was attested to be among those countries that are likely to achieve their goals. On the other hand, an overall lack of ambition was determined [56]: “Belgium did not go beyond existing standards at EU level”. In addition, there seemed to be no new assessments made specifically for the implementation of the MSFD and only little information on knowledge gaps was reported [56]. This suggests that while Belgium intends to cover chemical monitoring of and/or a reduction of the chemical pressure from EU priority pollutants,

neither CECs nor chemical mixtures will be addressed. In another report from the Commission to the European Parliament and the Council assessing the Member State's monitoring programs under the MSFD from 2017, the Belgian monitoring program was rated to be partially appropriate to meet the requirements of the MSFD and to partially measure progress towards the achievement of GES [57]. In concrete, it was recommended that Belgium should continue to integrate monitoring programs already existing under the WFD and enhance comparability and consistency of monitoring methods within its marine region by considering the monitoring scope, coverage, frequency and choice of indicators [57].

The above evaluations of the status of the Belgian compliance with the MSFD together with generally rising concerns about increasing input and presence of a broad range of chemicals into marine waters [8] are at the basis of this thesis. In addition, it appears that current monitoring practices under the MSFD in the BPNS solely focus on priority pollutants [56]. Not only does this approach neglect the well reported presence of CECs in marine waters but in addition, does it not provide any perspective for mixture-based risk assessment procedures that are needed to realistically assess the status of the BPNS from a chemical point of view.

## 1.7 Thesis outline and research objectives

This thesis made use of environmental samples taken with active and passive sampling at 4 sampling locations at the BPNS in 5 SCs between March 2016 and May 2018 (see Chapter 3.1). Active samples (water grab samples) were used as a reference point for the contaminants' occurrence and their realistic levels at the different sampling locations. However, no ecotoxicity tests were performed with grab samples, but the analytical results of the grab samples of SC1 served as a selection criterion for individual substance testing, which were aimed at filling data gaps on CECs ecotoxicity for marine species. This data was then merged with existing ecotoxicity data and used in an automated calculation algorithm programmed in R to derive screening-level PNECs for all CEC's. These PNECs were subsequently used in a single substance-based screening level risk assessment. In a next phase, we developed a MoS approach as a novel method to assess risks of ERCMs using passive sampler extracts. The MoS for a given species is to be considered as the number of times the ERCM occurring in the field can be enriched without resulting in significant toxicity.

The thesis is divided into four research chapters addressing the following topics.

### *1.7.1 Lack of ecotoxicological data for marine species*

It has generally been recognized that ecotoxicity data for marine species is less frequently available as compared to data for freshwater species [58]. This is largely due to the existence of fewer standard test guidelines for saltwater species and because especially European risk assessments historically tended to focus on freshwater systems [59]. The lack of knowledge for marine species frequently results in toxicity extrapolation from freshwater to saltwater species but this remains largely untested and the use of an additional AF of 10 is applied to marine risk assessments [59].

To address the ecotoxicity data gap for marine species, in **Chapter 4** a range of 23 different PCPs, pesticides and pharmaceuticals were tested individually in 72h algae growth inhibition

tests with *Phaeodactylum tricornutum* and in acute lethality tests with *Nitocra spinipes*. In addition, 7d larval development tests with *N. spinipes* were performed for four neonicotinoid insecticides that acutely showed to be the most toxic substances. Ultimately, EQS were derived for the four neonicotinoid insecticides and used together with monitoring data for SC2-5 in a risk assessment exercise. These tests and the subsequent risk assessment with neonicotinoid insecticides have been shown to be of great value since there is very limited information regarding the assessment of the toxic potential of neonicotinoid insecticides in marine ecosystems [60]. Also, monitoring and surveillance of neonicotinoid pollution of marine and coastal ecosystems seem to be non-existent [60].

### *1.7.2 Shift from priority pollutant to emerging chemical-focused risk assessment*

With the European Green Deal (EGD) presented in December 2019, the EU aims to i) boost the efficient use of resources by moving to a clean and circular economy and to ii) restore biodiversity and cut pollution. The latter goal shall be achieved by 2030 by fulfilling three key commitments: i) legally protect a minimum of 30 % of the EU's land area and 30 % of the EU's sea area and integrate ecological corridors, as part of the true Trans-European Nature Network, ii) strictly protect at least a third of the EU's protected areas, including all remaining EU primary and old-growth forests and iii) effectively manage all protected areas defining clear observation objectives and measures, and monitoring them appropriately [61]. With regards to the restoration of the GES of marine ecosystems, the EGD judges the full implementation of the MSFD as essential [61]. The overall ambition towards zero-pollution for a toxic free environment will be supported by the implementation of a chemicals strategy for sustainability (expected publication in summer 2020), a zero-pollution action plan for water, air and soil (2021) and a revision of measures to address pollution from large industrial installations (2021). A scientific approach towards a zero-pollution environment requires a thorough pollution survey by means of monitoring and risk assessment based on actual monitoring data. As such, **Chapter 5** was dedicated to the development of an automated calculation algorithm programmed in R to derive screening-level PNECs for all CECs detected in the NewSTHEPS project. Indeed, this covers a limited number of approximately 200 chemicals representing some of the most emerging chemical groups (i.e. personal care products, pesticides, pharmaceuticals, phenols, phthalates and steroids). Yet, in comparison with currently implemented monitoring studies focused on only 33 priority pollutants or pollutant groups our approach covers a wider range of chemicals with focus on rather emerging substances of potential concern. The PNECs derived with our automated method were subsequently compared to the monitoring data from the NewSTHEPS project to calculate screening-level risk quotients for a total of 65 substances. This approach offers a fast alternative for database-driven screening-level risk assessment that can be used as a tool for prioritization of CECs.

### *1.7.3 Development of a novel method for testing of ERCMs*

It is widely recognized that chemical pollution of the aquatic environment is a mixture rather than an individual substance issue. Chemicals follow many routes on their way into the different environmental compartments (***Figure 1.1***). Many chemicals find their way into the aquatic environments via small and medium sized streams (in rural regions) or the sewage system (in urban regions). Many of these are sooner or later connected to wastewater treatment plants where impurities including chemicals are intended to be removed. Here, removal efficiencies

for chemicals are usually identified based on chemical monitoring of specific target chemicals. This method not only risks to miss non-target chemicals and potential mixture effects of simultaneously present chemicals, it also has been shown to be unable to detect toxic potential of wastewater effluents [62]. In this scope, the need for effect-based monitoring tools has been increasingly emphasized in the past years [63-65].

While Chapter 4 and 5 thus focused on individual substances and individual substance-based risk assessment, **Chapter 6** describes the development of a novel method for passive sampler-based ecotoxicity testing of ERCMs. This method enables sample enrichment of ERCMs relative to their environmental concentration and allows testing in a (miniaturized) 72h algae growth inhibition test. Here, the method was applied to samples from the BPNS but the methodological principles are most likely also applicable for ecotoxicological monitoring of e.g. removal efficiencies of wastewater treatment plants.

### *1.7.4 Addressing marine environmental risks from a mixture perspective*

The method developed in Chapter 6 was first further improved to enable higher sample enrichment (needed to achieve effect-causing concentrations) and ultimately applied to samples from SC2, SC3 and SC5 in **Chapter 7**. The further method development allowed the definition of a MoS for the different SCs and locations. This MoS can be used as a tool for the identification of potential existing risks or help prioritizing (sampling) locations of emerging concern. It further allows to estimate a safety margin based on ecotoxicity testing of ERCMs which provides a novel approach for effect-based monitoring. Many effect-based monitoring approaches so far focus on steroids or endocrine activity [62, 66] and use mainly specific *in-vitro* assays to assess the toxic potential of sampled chemical mixtures. In our study, chemical analysis focused on personal care products, pesticides and pharmaceuticals. This different perspective together with the use of *in-vivo* testing with algae who represent a higher level of biological organization as compared to *in-vitro* bioassays may thus be seen as complementary alternative to *in-vitro* testing of ERCMs. An overview of the main research chapters and how their conjunction is given in **Figure 1.3**.

The overall scope of this research are the identification of CECs for prioritization in agreement with current legislative frameworks and the development of an effect-based monitoring approach for ERCMs ready-to-use as mixture-based environmental risk assessment approach for the marine environment. To achieve these objectives, four specific research goals were defined:

1. The **identification and prioritization of CECs** in the BPNS.
2. The development of an automated approach for **screening-level risk assessment for CECs** as complementary tool for marine environmental monitoring.
3. The **development of an effect-based monitoring** approach for testing of **ERCMs**.
4. **Execution of a mixture-based environmental risk assessment for the BPNS** and comparison with prevailing single substance-based risk assessment approaches.

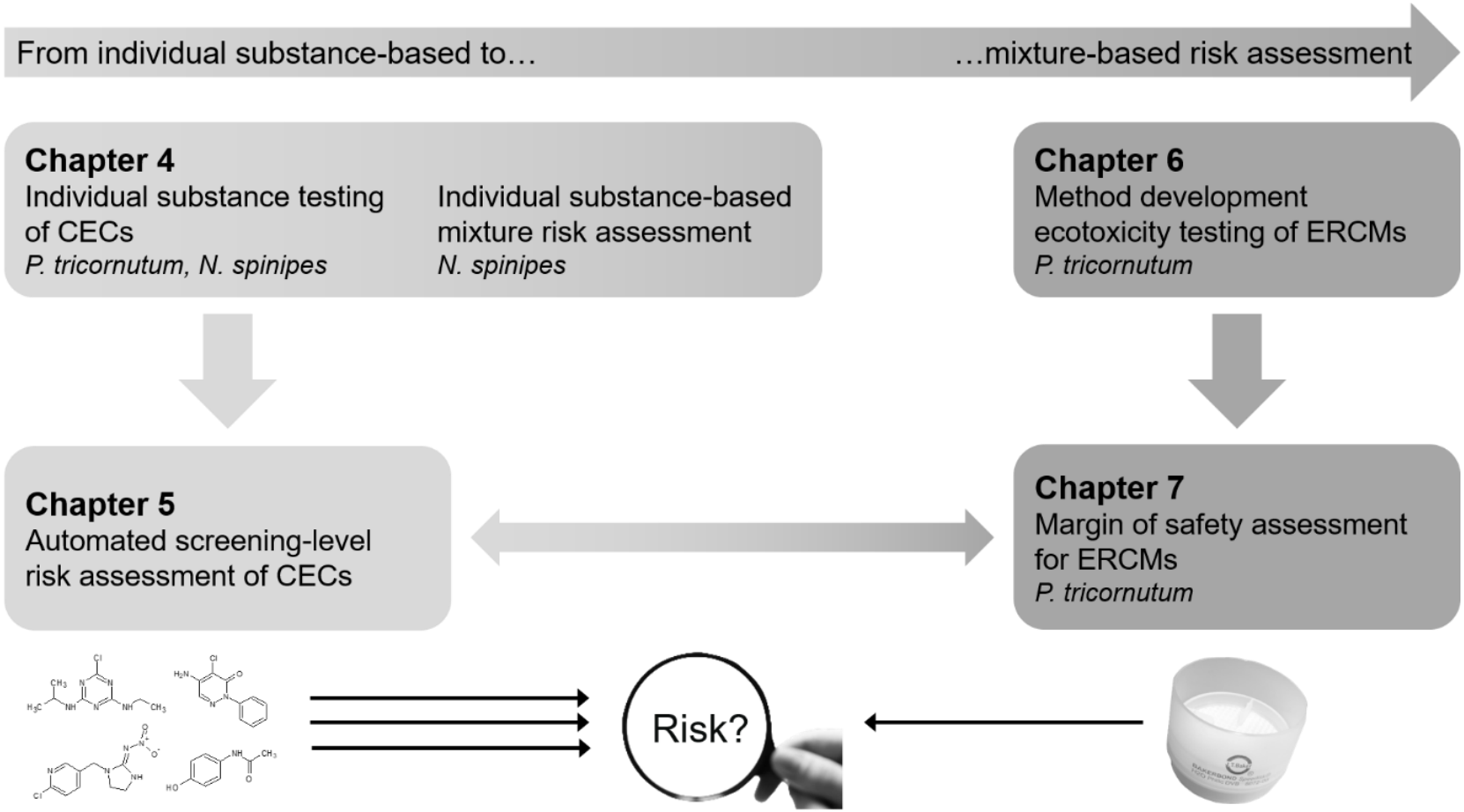



**Figure 1.3 Conceptual framework of the four main research chapter of this thesis. Individual substance testing for chemicals of emerging concern (CECs) was used as basis for an individual substances-based risk assessment for the Belgian Part of the North Sea (BPNS) (Chapter 4). The results of Chapter 4 were complemented by literature data and used in an individual substance-based automated screening-level risk assessment for CECs (Chapter 5). In Chapter 6 a method for passive sampler-based testing of environmentally realistic chemical mixtures (ERCMs) was developed and applied in an explorative way for samples from sampling campaign 1 and 4 (SC1 and SC4). Finally, Chapter 7 describes how the previously developed method was adjusted to achieve higher sample enrichment and applied to samples from SC2, SC3 and SC5 to derive a Margin of Safety for ERCMs from the BPNS.**

# 2

# Test organisms and biotest systems

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# 2 Test organisms and biotest systems

## 2.1 Test organisms

All ecotoxicity testing in this research was performed with two model species: the marine diatom algae *Phaeodactylum tricornutum* and the brackish harpacticoid copepod *Nitocra spinipes*.

*Phaeodactylum tricornutum Bohlin* (see ***Figure 2.1***) is a marine diatom algae and was obtained from the Culture Collection of Algae and Protozoa (CCAP 1052/1A, Oban, United Kingdom). Algae were cultured in marine algae growth medium according to the ISO 10253 guideline (ISO, 2006). All details about the medium composition and general culturing conditions can be found in this guideline. Four days prior to a test initiation, a pre-culture was prepared by inoculating fresh growth medium with 10,000 cells $mL^{-1}$. Both the culture and pre-culture were allowed to grow under continuous white light (100 -120 µmol $m^{-2}$ $s^{-1}$) at 22 ± 1 °C.

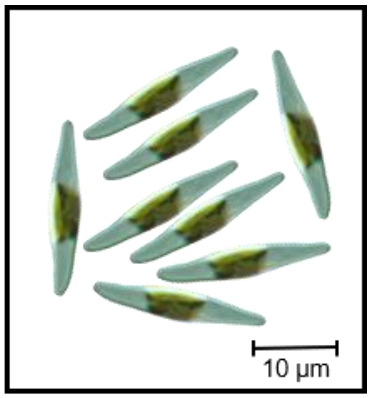



**Figure 2.1 *Phaeodactylum tricornutum* Bohlin, 1897.**

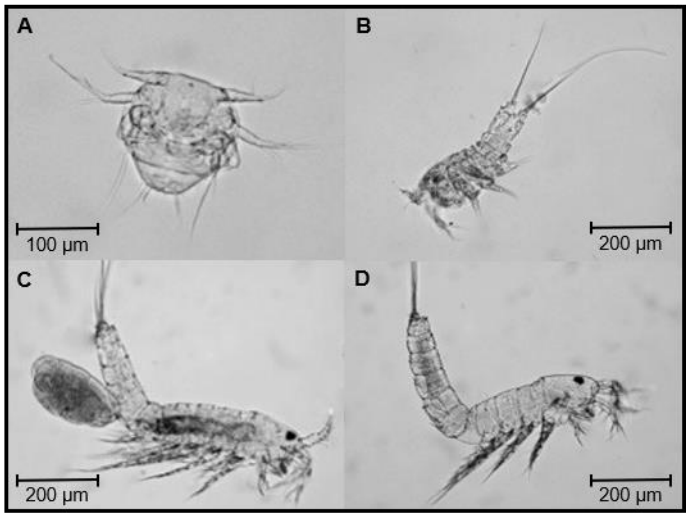



**Figure 2.2 *Nitocra spinipes* Boeck, 1865. Shown are the 3 major morphological stages, nauplius (A), copepodite (B) as well as egg-carrying female (C) and male (D) adults. © Josef Koch**

*Nitocra spinipes Boeck* (see ***Figure 2.2***) is a harpacticoid copepod and was obtained from the Department of Environmental Science and Analytical Chemistry (ACES) at Stockholm University, Sweden, where *N. spinipes* has been in continuous culture since 1975 when it was isolated from the Tvären Bay in the Baltic Sea. A culture was established in our laboratory in 2016 (Koch and De Schamphelaere 2019). *N. spinipes* is cultured according to methods described in Breitholtz and Bengtsson (2001) in natural seawater diluted to a salinity of 7 ‰ using deionized water. Natural seawater was collected 500 m off the coast of Blankenberge, Belgium and filtered using 0.2 µm PALL Supor®-200 membrane filters. The culture was permanently maintained in darkness at 21 ± 1°C.

## 2.2 Biotest systems

In total, we applied four biotest systems, two each for algae and copepods.

**Biotest system 1** was the standardized 72h growth inhibition test with *P. tricornutum* according to ISO 10253 [67]. Here, *P. tricornutum* is exposed to a test compound (or a mixture) in an erlenmeyer flask containing 50 mL algae growth medium (preparation described in the guidance). Algae were usually exposed to a dilution series of at least 6 concentrations following a dilution factor. Each concentration treatment (CT) was tested in triplicate erlenmeyer flasks containing algae test medium, a specific concentration of the test substance (or mixture) and inoculated with 10,000 cells $mL^{-1}$ at test start. Additionally one erlenmeyer flask per CT was filled with algae test medium and test substance (or mixture) but not inoculated to serve as particle blank. At least 6 erlenmeyer flasks per test were filled with algae growth medium and inoculated with 10,000 cells $mL^{-1}$ to serve as controls (without added compound or mixture). Test flasks were cultivated under the same conditions as the general culture and pre-cultures and shaken manually twice a day. Cell density in all flasks was measured daily over 72h using an electronic particle counter (Beckman Z2™ Coulter Counter® Analyzer). The specific growth rate μ was calculated for the different controls and each CT by applying the SLOPE function (Excel 2016) on the Ln-transformed cell counts of the measurements of day 0 to day 3. The percentage of growth inhibition (I) for each replicate test flask ($I_{\mu,i}$) was then calculated as follows:

$$I_{\mu,i} = \frac{\overline{\mu_c} - \mu_i}{\overline{\mu_c}} * 100 \qquad \textbf{(Eq. 2.1)}$$

with $\mu_c$ the growth rate [$d^{-1}$] of the controls and $\mu_i$ as the growth rate [$d^{-1}$] of the individual test flasks. For the identification of differences in growth rates between concentrations and controls, one-way analysis of variance (one-way ANOVA, α = 0.05) was applied, followed by Dunnett's multiple comparisons test. This analysis was performed in GraphPad Prism version 5.01 for Windows (Muzyka, Tarkany et al. 2007).

**Biotest system 2** was a modified version of the 72h growth inhibition test with *P. tricornutum*. This adaptation was a result of the method development in chapter 5. Instead of performing the algae growth inhibition tests in erlenmeyer flasks containing 50 mL algae growth medium, the tests were performed in 24-well plates containing 2 mL algae growth medium per well. Cultivation conditions were identical with the addition of constant shaking of the test plates at 120 rpm. In addition, fluorescence measurements (using a TECAN® Infinite M200 PRO) were performed instead of cell counting to monitor algae growth. Settings for the measurement of a 24-well plate inoculated with *P. tricornutum* are shown in ***Table 2.1***. In addition to triplicate wells per CT, 3 wells per plate were filled with 2 mL growth medium and inoculated with *P. tricornutum* to serve as controls and 6-9 wells per test setup were filled with growth medium but not inoculated to serve as particle blanks. In the beginning of a test, a calibration series ranging from 10,000 to 400,000 cells $mL^{-1}$ based on cell counts (see Biotest system 1) was prepared and fluorescence measured at test start. This calibration series was used to subsequently convert the fluorescence measurements to cell counts. After conversion, growth rates and growth inhibition were calculated as described in Biotest system 1. Statistical analysis was also performed as described in Biotest system 1.

**Table 2.1 Settings for fluorescence measurements of 24-well plates inoculated with *P. tricornutum* using a TECAN Infinite M200 PRO**

| Factor | Setting |
|---|---|
| Shaking duration | 5 s |
| Shaking amplitude | 2.5 mm |
| Shaking direction | Orbital |
| Mode | Fluorescence top reading |
| Excitation wavelength | 488 nm |
| Emission wavelength | 700 nm |
| Excitation bandwidth | 9 nm |
| Emission bandwidth | 20 nm |
| Gain | Fixed (as determined by calibration series) |
| Number of reads | 25 |
| Integration time | 20 µs |
| Lag time | 0 µs |
| Settle time | 0 ms |

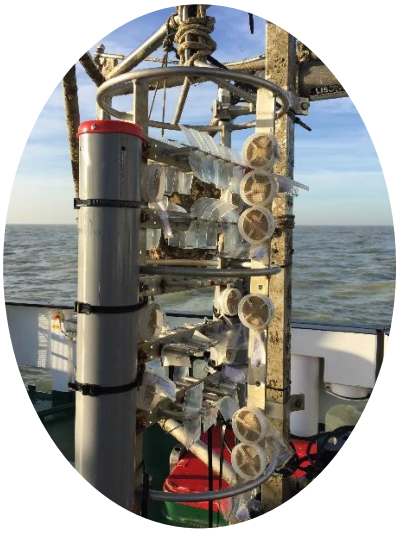
**Biotest system 3** was the standardized acute lethal toxicity test to marine copepods with *N. spinipes* according to ISO 14669 [68]. In short, 3-4 week old copepods were exposed to different concentrations of selected chemicals and mortality was monitored daily over 96h. In addition, immobility was recorded and defined as no swimming (no controlled vertical or horizontal movement) of copepods during 10 seconds, whereas mortality was defined as no swimming and no appendages movement during 10 seconds such as described in ISO 14669 [68]. During the test, organisms were kept in darkness at 21 ± 1°C such as the cultures. For each CT, 20 organisms were randomly selected regardless of gender (including egg-carrying females) and separated into 4 replicates of 5 organisms. In addition, 24 wells distributed over different plates were filled with pure diluted natural seawater and 5 copepods each to serve as control treatments. They were exposed in 2.5 mL test medium spiked with the respective test substance in sterilized non-treated 24-well VWR® (Oud-Heverlee, Belgium) cell culture plates. Test concentrations were determined in a previously performed range-finding test in which 5 adult copepods were exposed to six concentrations ranging from 0.01 to 100 mg $L^{-1}$ in a geometric concentration series with spacing factor 10. For the definite test, substances were dissolved in diluted seawater using ultra sonication if needed and a geometric dilution series was prepared directly in the well plates. pH was measured in one well of the lowest and highest test concentrations of each substance as well as two control wells at test start and end.

**Biotest system 4** was the standardized larval development test with the harpacticoid copepod *Nitocra spinipes* according to ISO 18220 [69]. In short, nauplii younger than 24h from *N. spinipes* were exposed to six different CTs of the four neonicotinoid insecticides clothianidin (CLO), imidacloprid (IMI), thiacloprid (TCP) and thiamethoxam (TMX). Naupliar development to the copepodite stage was recorded after 6 and 7 days of exposure, and expressed as larval development ratio (LDR) being the number of copepodites divided by the sum of nauplii plus copepodites. At test start, 9 ± 1 nauplii were placed in eight replicate wells per concentration or control treatment for CLO, IMI, and TMX. Because of a limited availability of nauplii at test start of TCP only 6 ± 1 nauplii were used in the replicates and controls for TCP. Seventy percent of the test medium was refreshed once on day 4 and pH and salinity were measured

at test start, day 4 and day 7. Organisms were exposed in 10 mL test medium spiked with the respective test substance (or not spiked in case of control treatments) in sterilized non-treated 6-well VWR® (Oud-Heverlee, Belgium) cell culture plates. During the tests, organisms were constantly kept in darkness at 21 ± 1°C as the cultures.

# 3

# Sampling in the Belgian part of the North Sea



# 3 Sampling in the Belgian part of the North Sea

## 3.1 Active and passive sampling

Within the NEWSTHEPS project, five field sampling campaigns (SCs) were performed. Samples were taken at four different sampling locations, i.e. inside the harbor of Zeebrugge (HZ, 51°20'25.68"N; 3°12'12.11"E) and a few kilometers off the coast of Zeebrugge harbor (OZ_MOW1, 51°21'37.78"N; 3°6'49.01"E), and inside the harbor of Ostend (HO, 51°13'34.68"N; 2°56'8.00"E) and a few kilometers off the coast of this harbor (OO_X, 51°15'33.00"N; 2°58'1.20"E). An overview of the sampling locations is depicted in ***Figure 3.2***. Sampling locations inside the harbors were selected to represent high shipping activities and due to simple accessibility. Coastal locations were selected to represent open marine waters and were sampled using the research vessel Belgica A962.

Five SCs were carried out between March 2016 and May 2018. Details about the different SCs can be found in ***Table 3.1***. At the beginning and end of each SC triplicate grab water samples were taken. For this purpose, 3000 mL water was collected and divided into 3 sub-samples of 1000 mL each. As such, these sub-samples cannot be considered as real sampling replicates but rather as analytical replicates (3 analytical measurements of one grab sample). This was mainly due to limited time at the respective sampling locations since our sampling campaigns were usually part of joint expeditions at the BPNS. Grab water samples were stored in amber glass bottles pre-cleaned with methanol and ultrapure water. Upon arrival in the laboratory, sub-samples were filtered using Whatman GF/D glass fiber filters (2.7 µm, 90 x 90 mm). $Na_2EDTA{\cdot}2H_2O$ at a concentration of 1 g $L^{-1}$ was added to the water grab samples and the pH was adjusted to 7 by addition of a 10 % formic acid solution. Then, samples were stored at 4 °C and dark conditions until extraction and analysis. Finally, chemicals were extracted from the samples by means of solid-phase extraction (SPE) using Oasis Hydrophilic-Lipophilic Balance (HLB) (6 mL, 200 mg sorbent, Waters, Belgium).

Passive sampling was performed using Bakerbond $H_2O$-philic divinylbenzene speedisks (Filter Service S.A, Eupen, Belgium). Speedisks have widely been used for solid-phase extraction in water analysis [70]. They consist of hydrophilic divinylbenzene sorbent embedded between two glass fiber filters, held together by two screens, topped by a retaining ring and incorporated into a housing (***Figure 3.1***). For use as passive sampling device, the commercially available Speedisk was physically manipulated by removing the top of the housing with the intention to improve water flow towards the sorbent. In addition, four holes were drilled on the bottom of the housing to allow fixing of the speedisk. No changes to the sorbent or fixing parts of the sorbent were made to maintain integrity of the sampling phase.

During sampling, a total number of 16 speedisks were deployed per sampling location. Field blank passive samplers (handled equally to the deployed speedisks) were included to assess potential contamination during handling and transport. Before deployment, the samplers were pre-rinsed with 20 mL HPLC grade methanol : HPLC grade acetonitrile (1:1, v/v %), subsequently with 20 mL HPLC water and finally stored in acid-washed glass bottles filled to the top with HPLC grade water until deployment. Samplers were deployed in triplicates at a depth of approximately 3m below the surface at the harbors and above 2m from the seabed at the coastal locations. After retrieval they were immediately stored in empty glass bottles and

kept in the dark at 4 °C until extraction. Speedisks were rinsed directly after recovery with water of the respective sampling location. In the lab, speedisks were extracted following three steps:

1. Rinsing of speedisks by passing 18 mL HPLC water over the sorbent phase.
2. Drying of the sorbent under vacuum for 5 min.
3. Extraction using 10 mL HPLC grade methanol : acetonitrile (1:1, v/v).

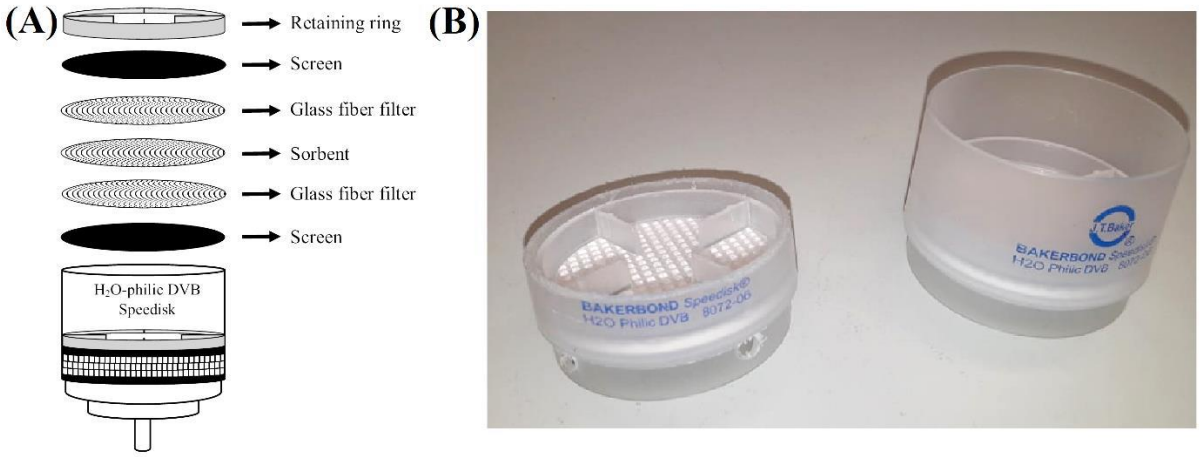



**Figure 3.1 Schematic representation of the configuration of the hydrophilic divinylbenzene Speedisk (A). The picture on the right shows the manipulation of the Speedisk including removal of the top housing and drilling of fixing holes (B) [71].**

Subsequent treatment is described in detail in Chapters 6 and 7 and differed along the method development. Details about the analytical procedures can be revised in Vanryckeghem et al. (2019) [20]. In general, within the scope of this thesis, chemical analysis of environmental samples focused on the detection and quantification of 89 personal care products (PCPs), pesticides and pharmaceuticals (see annex A, Table A1). These substances were selected based on their occurrence in natural waters, high consumption/usage and legislative frameworks such as e.g. the Water Framework Directive (WFD) Watchlist [72].

**Table 3.1 Overview of the sampling campaigns (SC) at the harbors of Zeebrugge (HZ) and Ostend (HO) and at the coast of Zeebrugge (OZ_MOW1) and Ostend (OO_X). Grab samples were taken at Speedisks passive sampler deployment and retrieval. NA means no samples were taken due to unavailability of the research vessel. All grab water samples were taken and speedisks were deployed in triplicates.**

| SC | Location | Start | End | Deployment | Retrieval | Speedisks |
|---|---|---|---|---|---|---|
| SC1 | HZ | 14/03/2016 | 20/05/2016 | YES | YES | YES |
| | HO | 14/03/2016 | 20/05/2016 | YES | YES | YES |
| | OZ_MOW1 | 14/03/2016 | 20/05/2016 | YES | YES | YES |
| | OO_X | NA | NA | NA | NA | NA |
| SC2 | HZ | 25/11/2016 | 02/02/2017 | YES | YES | YES |
| | HO | 25/11/2016 | 02/02/2017 | YES | YES | YES |
| | OZ_MOW1 | 23/11/2016 | 06/02/2017 | YES | YES | YES |
| | OO_X | 23/11/2016 | NA | YES | NO | NO |
| SC3 | HZ | 13/04/2017 | 20/06/2017 | YES | YES | YES |
| | HO | 13/04/2017 | 20/06/2017 | YES | YES | YES |
| | OZ_MOW1 | 23/05/2017 | 14/07/2018 | YES | NO | YES |
| | OO_X | 02/05/2017 | 26/07/2017 | YES | YES | YES |

| | | | | | | |
|---|---|---|---|---|---|---|
| SC4 | HZ | 16/10/2017 | 18/12/2017 | YES | YES | YES |
| | HO | 16/10/2017 | 18/12/2017 | YES | YES | YES |
| | OZ_MOW1 | 26/10/2017 | 19/12/2017 | YES | YES | YES |
| | OO_X | 26/10/2017 | 10/04/2018[a] | YES | YES | NO |
| SC5 | HZ | 29/03/2018 | 17/05/2018 | YES | YES | YES |
| | HO | 29/03/2018 | 17/05/2018 | YES | YES | YES |
| | OZ_MOW1 | 29/03/2018 | NA | YES | NO | NO |
| | OO_X | 29/03/2018 | NA | YES | NO | NO |

[a] Due to loss of the tripod, no passive samplers were retrieved at this time-point.

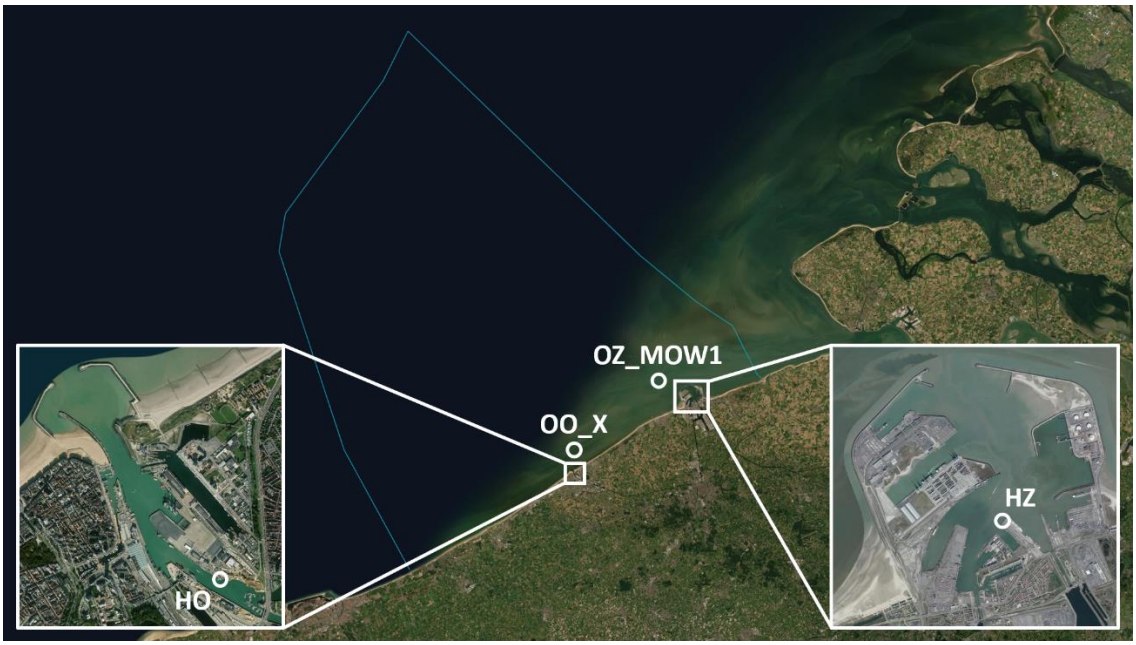



**Figure 3.2 Map of the Belgian coast indicating the four sampling locations. HO and OO_X stand for "harbor Ostend" and "coastal sampling location near Ostend", respectively while HZ and OZ_MOW1 stand for "harbor Zeebrugge" and "coastal sampling location near Zeebrugge". The blue line shows the borders of the Belgian part of the North Sea.**

## 3.2 Chemical analytical methodology

All chemical analysis was performed as described in Vanryckeghem (2020) [71]. In short, chemical analysis was performed by injection of 10 µL of the reconstituted extracts (see section 3.1) on a reversed phase ultra-high performance liquid chromatography (UHPLC) system equipped with a Hypersil Gold column (1.9 µm particle diameter, 2.1 x 50 mm, Thermo Scientific). The mobile phase was operated at a flow rate of 350 µL $min^{-1}$ (Accela 1250 pump, Thermo Scientific) and consisted of a biphasic gradient using water and methanol both acidified with 0.1 % and 0.01 % formic acid for the positive and negative ionization mode, respectively. The UHPLC was coupled to a hybrid quadrupole-Orbitrap high-resolution mass spectrometer (Q-Exactive™, Thermo Scientific) equipped with a heated electrospray ionization source and operating in full scan mode (120–760 *m/z* for HESI-positive; 100–760 *m/z* for HESI-negative) at a resolving power of 70,000 at full width at half maximum at 200 *m/z*. The optimal HESI-II parameters were: capillary temperature: 350 °C; heater temperature: 375 °C; spray voltage: ± 3.5 kV; sheath gas flow rate: 45 absolute units; auxiliary gas flow rate: 10 AU; S-lens RF-level: 60%. The automatic gain control target was set at 3,000,000 ions with a maximal injection time of 200 ms.

# 4

# Single substance testing of chemicals of emerging concern

Redrafted from:

Moeris, S., Vanryckeghem, F., Demeestere, K., De Schamphelaere, K.A.C. Neonicotinoid insecticides from a marine perspective: Acute and chronic copepod testing and derivation of Environmental Quality Standards. Environmental Toxicology and Chemistry, under review.

# 4 Single substance testing of chemicals of emerging concern

## 4.1 Introduction

Before importing or placing on the European market (above 1 ton per year) a new chemical needs to be tested to investigate its physico-chemical properties as well as potential hazards for human and environmental health [12]. Individual substance testing is thus at the basis of current day's regulatory requirements. As such, data generation is crucial in order to predict potential hazards to the environmental and/or human health. Still, most of the data requirements are limited to specific standard biotest organisms often excluding marine species. This resulted in a considerable discrepancy between ecotoxicity data for freshwater and saltwater species especially for organic compounds [59]. Remarkably, derivation of environmental threshold values for the marine environment such as e.g. Environmental Quality Standards (EQS) can be solely based on ecotoxicity data for freshwater species [6]. This might be one reason as to why the availability of marine ecotoxicity data is certainly less than optimal for marine environmental risk assessment [7].

About 20 % of the Earth's photosynthesis is carried out by diatoms [73]. *Phaeodactylum tricornutum* is an example for a diatom that has become a true model organism in environmental research proven by the availability of its whole-genome sequence [74]. *P. tricornutum* are important and widely distributed phytoplankton species found worldwide in estuarine and coastal areas [67].

On a higher trophic level, copepods have been found to play an important role in the Belgian Part of the North Sea (BPNS) where they comprise 66 % of the total zooplankton abundance [75] serving as prey for higher trophic levels [76]. Over the past two decades the harpacticoid copepod *Nitocra spinipes* has become a standard test species representing coastal and estuarine organisms [77, 78], resulting in the development of international acute and chronic ecotoxicity testing guidelines [68, 69].

An initial literature screening for ecotoxicity data for marine species revealed that for 89 target personal care products (PCPs), pesticides and pharmaceuticals data availability is mainly restricted to WFD priority pollutants (e.g. atrazine, cybutryne or diuron) and other well studied substances. In this study we identified and filled data gaps for marine species by acutely testing 23 chemicals of emerging concern (CECs) with both the marine diatom *P. tricornutum* and the brackish copepod *N. spinipes*. Based on the acute toxicity we chronically tested four neonicotinoid insecticides with *N. Spinipes* and derived EQS based on a combination of our generated and literature ecotoxicity data. Ultimately, we characterized potential risks for the BPNS for individual neonicotinoids and their mixture.

## 4.2 Materials and methods

### *4.2.1 Compound selection*

This chapter focused on PCPs, pesticides and pharmaceuticals from the list of NewSTHEPS target compounds (annex A Table A1). Compound selection for ecotoxicity testing was based on:

1. Analytical results of grab water samples from SC1 (see section 3): All substances without any detection above the method detection limit (MDL) were excluded.
2. Ecotoxicity data for marine crustaceans: Substances with ecotoxicity data for both marine algae and crustaceans available in the EPA ECOTOX database (US Environmental Protection Agency 2019) were excluded since the main goal was to fill data gaps for marine species.
3. Log $K_{OW}$: Substances with a log $K_{OW}$ > 3 were excluded with four exceptions, i.e. alachlor due to its detection frequency of 100 %, bezafibrate being the only representative of the sub-class of lipid-regulating pharmaceuticals, diclofenac due to high measured concentrations up to 269 ng $L^{-1}$ and venlafaxine as representative of an antidepressant with high usage. Other substances were excluded because a higher log $K_{OW}$ can be associated with demanding test setups such as passive dosing to maintain constant exposure conditions [79].
4. Price: some substances (bisoprolol, gatifloxacin and sotalol) were excluded due to very high costs (>850 € per g) for the pure substance.

All substances were purchased from Sigma-Aldrich (Overijse, Belgium). An overview of the substances selected for biotesting is given in annex A (Table A1).

### *4.2.2 Biotest systems*

The respective CECs were tested with Biotest systems 1 and 3 for *P. tricornutum* and *N. spinipes*, respectively (see section 2.2). Four neonicotinoid insecticides were found to be the most acutely toxic for *N. spinipes* and were therefore also tested (sub-)chronically using Biotest system 4. Sub-chronic toxicity testing with *N. spinipes* was limited to the four neonicotinoid insecticides since no other investigated chemical showed comparably high toxicity when tested acutely. Indeed, there is a possibility that a substance that is not acutely toxic might exert long-term effects but testing all 23 substances chronically was not feasible within this thesis. Therefore a selection was made based on acute toxicity testing.

For algae growth inhibition testing, the temperature was measured continuously throughout the test period in an additional erlenmeyer flask filled with test medium. The pH was measured daily in one randomly picked flask per concentration treatment (CT).

#### *4.2.3 For acute lethality testing with* N. spinipes*, a pre-test was performed exposing 5 adult copepods to 6 CTs ranging from 0.001 – 100 mg $L^{-1}$. For those substances that did not exert any toxicity on* N. spinipes *up to 100 mg $L^{-1}$, a limit tests was subsequently performed by exposing 20 adult copepods to a nominal concentration of 100 mg $L^{-1}$. pH and salinity was measured in one well of the lowest and highest test concentrations of each substance as well as two control wells at test start and end.Derivation of Environmental Quality Standards*

EQS (equivalent to predicted no-effect concentration, PNEC) were derived according to the technical guidance document (TGD) for deriving EQS [6] using the AF method. Toxicity data was obtained from two databases: the US Environmental Protection Agency (EPA) ECOTOX database [80] and the US EPA Office of Pesticide Programs (OPP) Pesticide Ecotoxicity database [81]. More information about the two databases can be found in annex A.

### *4.2.4 Chemical analysis and statistical evaluations*

#### *4.2.4.1 Growth inhibition testing with P. tricornutum*

Samples of the growth medium (control and contaminant-spiked) for analysis were taken at the beginning and end of each test. In detail, 225 mL per concentration treatment (CT) were sampled at the beginning of each test and stored at -20 °C until extraction. At the test end, triplicates were merged and filtered using a Whatman GF/D glass fiber filter (2.7 µm pore size). After filtering, 100 mL per CT were stored at -20 °C until extraction for chemical analysis.

Dose-response models were generated using the “drc” package [82] using a four-parameter log-logistic model (fct = LL.4, logDose = 10). $EC_{10}$ and $EC_{50}$ values with their 95 % confidence intervals (CI) were derived from the model.

#### *4.2.4.2 Acute lethality testing with N. spinipes*

Samples of the test medium (control and contaminant-spiked) for analysis were taken at the beginning and end of the tests. More precisely, at test start 1 mL of each stock solution as well as the different CTs were taken for each substance. Samples from individual chemical tests were merged per CT and stored in amber glass bottles at darkness and -20 °C until analysis. At test end, 250 µL were taken from each replicate well and merged in an amber glass bottle. Next, the corresponding CTs of each individual test were merged i.e. CT1 of the different individual substance tests were merged in one bottle, CT2 of the different individual substance tests in a second bottle etc. All samples were filtered using 2.7 µm glass microfiber filters (Whatman™ GF/D, GE Healthcare) before storage in darkness at -20 °C.

Statistical analysis was performed in R Studio [83]. Dose response models were generated using the “drc” package [82] and visualizations were created with ggplot2 [84]. For dose-response analysis, a two-parameter log-logistic model was used (fct = LL.2 (upper = 100), logDose = 10). $EC_{10}$ and $EC_{50}$ values with their 95 % CI were derived from the model.

#### *4.2.4.3 Larval development testing with N. spinipes*

Known volumes (90 mL, 56 mL and 75 mL) of the test medium were taken at day 0 (test start), day 4 and day 7 of each test. Samples taken on day 4 consisted of 7 mL taken from each of the 8 replicate wells of each CT. Samples at day 0 were samples taken directly from the prepared stock solution. Samples at day 7 were a combination of the remaining test medium in all 8 replicate wells of each CT. Samples from CT and the controls were then filtered using 2.7 µm glass microfiber filters (Whatman™ GF/D, GE Healthcare) and stored in amber glass bottles in darkness at -20 °C until analysis.

Statistical analysis was performed in R Studio [83]. The larval development ratio (LDR) was defined as the ratio of copepodites to the total number of surviving early-life stages (nauplii + copepodites) at the end of the test. Statistical analysis was performed using non-parametric tests. Differences between specific treatments and the control were assessed using the Mann-Whitney-U test. Based on this analysis, the no-observed effect concentration (NOEC) for each substance was defined as the highest concentration showing no statistically significant (Mann-Whitney-U-Test, $\alpha = 0.05$) effect on larval development. In addition to that, the lowest-observed effect concentration (LOEC) was defined as the lowest concentration showing a statistically significant (Mann-Whitney-U-Test, $\alpha = 0.05$) effect on larval development. In a separate analysis, a concentration-response model was fitted in R Studio using the "drc" package [82] to determine the $EC_{10}$. For clothianidin (CLO), a hormetic four-parameter model (CRS.4c) was fitted to the data. For imidacloprid (IMI) and thiacloprid (TCP) a log-logistic two-parameter model (llogistic2) was used where the maximum LDR was set to the observed average LDR of the control treatment. For thiamethoxam (TMX), no effects were observed and thus no model fitted.

#### *4.2.4.4 Risk characterization*

Risks for the BPNS were calculated for all neonicotinoids using the toxic unit (TU) approach. For each substance an individual TU was calculated as the ratio between the measured concentration of the substance and its annual average (AA)-EQS for saltwater environments. For the mixture risk assessment, the individual neonicotinoid TUs were summed (∑TU) per sampling location ***(Eq. 4.1)***. A $TU_i > 1$ or $\sum_{i=1}^{n} TU_{i,x} > 1$ indicates a risk of the individual substance or the mixture, respectively (i.e., RQ >1). The RQ of the mixture ($RQ_{mix}$) was calculated as follows:

$$RQ_{mix} = \sum_{i=1}^{n} TU_{i,x} = \sum_{i=1}^{n} \frac{C_i}{EQS_i} \qquad \textbf{(Eq. 4.1)}$$

where n is the number of mixture components considered and $TU_i$ is the TU of component *i* in the mixture. The $TU_i$, a dimensionless parameter, is defined as the ratio between concentration of component *i* in the mixture ($C_i$) and its AA-EQS for saltwater environments in the mixture ($EQS_i$).

## 4.3 Results

### *4.3.1 Growth inhibition testing with P. tricornutum*

***Table 4.1*** shows the results of the single substance testing of the selected CECs with the marine diatom *P. tricornutum*. The lowest $EC_{50}$ (72h) was observed for amantadine and oxytetracycline with 6.6 mg $L^{-1}$ and the lowest $EC_{10}$ (72h) for oxytetracycline with 0.29 mg $L^{-1}$.Overall, these data suggest a low sensitivity (>0.1 mg $L^{-1}$) of *P. tricornutum* to these substances. A summary of the test concentrations and pH measurements for the lowest and highest concentration treatments is given in annex A (Table A2).

**Table 4.1 Effect concentrations (mg $L^{-1}$, based on the average measured concentrations of triplicates) and lower and upper 95% confidence limits (CL) of individual emerging micropollutants determined in the 72h growth inhibition tests with Phaeodactylum tricornutum. $EC_{10}$ = 10% effective concentration; $EC_{50}$ = 50% effective concentration. NA indicates not applicable, NM not measured.**

| Substance | $EC_{10}$ | Lower CL | Upper CL | $EC_{50}$ | Lower CL | Upper CL | ΔpH | ΔT (°C) |
|---|---|---|---|---|---|---|---|---|
| Alachlor | 3.6 | 1.2 | 6.0 | 20 | 14 | 25 | 0.88 | NM |
| Amantadine | 3.5 | 2.9 | 4.1 | 6.6 | 5.7 | 7.4 | 0.88 | NM |
| Atenolol[a] | >100 | NA | NA | >100 | NA | NA | 0.64 | 1.8 |
| Bezafibrate | >100 | NA | NA | >100 | NA | NA | 0.39 | 1.6 |
| Carbamazepine | 46 | 34 | 57 | 84 | 78 | 90 | 0.80 | 0.90 |
| Chloridazon | 0.58 | 0.08 | 1.07 | 9.7 | 5.2 | 14 | 0.67 | 0.90 |
| DEET | >53 | NA | NA | >53 | NA | NA | 0.88 | NM |
| Diclofenac[a] | >100 | NA | NA | >100 | NA | NA | 0.39 | 1.6 |
| Flufenacet | 7.0 | 3.7 | 10 | 23 | 19 | 26 | 0.57 | 0.80 |
| Flumequine[a] | >100 | NA | NA | >100 | NA | NA | 0.25 | 1.2 |
| Imidacloprid | >160 | NA | NA | >160 | NA | NA | 0.91 | NM |
| Mecoprop[a] | >100 | NA | NA | >100 | NA | NA | 0.40 | 1.6 |
| Metoprolol | 0.40 | 0.06 | 0.73 | 7.6 | 0.8 | 14 | 0.25 | 1.2 |
| Moxifloxacin | 23 | 10 | 36 | 126 | 95 | 157 | 0.25 | 1.2 |
| Oxytetracycline | 0.29 | 0.09 | 0.66 | 6.6 | 0.9 | 14 | 0.25 | 1.2 |
| Pirimicarb | >148 | NA | NA | >148 | NA | NA | 0.52 | 0.90 |
| Sulfamethoxazole | >323 | NA | NA | >323 | NA | NA | 0.45 | 0.90 |
| Thiacloprid | 42 | 34 | 50 | 103 | 96 | 111 | 0.60 | 0.80 |
| Venlafaxine | 6.9 | 3.0 | 11 | 19 | 13 | 24 | 0.65 | 0.80 |
| Zidovudine[a] | >100 | NA | NA | >100 | NA | NA | 0.67 | 0.80 |

[a]Based on nominal concentration

### *4.3.2 Acute lethality testing with N. spinipes*

The pH across all measurements within a test varied maximally 1.7 units (9.1 – 7.4, for amantadine) and on average 0.4 units. The complete pH data can be found in annex A (Table A3). In addition to the standard endpoint mortality, immobilization was also monitored for all substances and observed it for the four neonicotinoid insecticides. Immobilization resulted in clearly lower (2.6 – 1000 times lower) 96h-$EC_{50}$ values as compared to mortality. **Table 4.2** shows the $EC_{50}$ values of the four neonicotinoid insecticides including their CIs. The differences between mortality and immobilization $EC_{50}$ values were generally larger after short exposure times (24h – 48h). Immobilization $EC_{50}$ (96h) values were 2.6, 6.2, 847 and 1000 times lower

than the mortality $EC_{50}$ (96h) for CLO, TMX, TCP and IMI, respectively. The lowest immobilization $EC_{50}$ (96h) were observed for CLO at 6.9 µg $L^{-1}$ and TCP at 7.2 µg $L^{-1}$, whilst the observed effects for CLO and TMX became increasingly comparable for the two endpoints (mortality and immobilization) over time. This was not the case for IMI and TCP where mortality was a clearly less sensitive endpoint than immobilization even after 96h as shown in **Figure 4.1**.

For substances other than the neonicotinoids, $EC_{50}$ (96h) values were determined for amantadine at 4.8 mg $L^{-1}$ (3.0 – 6.6 mg $L^{-1}$), alachlor at 12 mg $L^{-1}$ (95%-CI = 11 – 13 mg $L^{-1}$), diclofenac at 21 mg $L^{-1}$ (19 – 23 mg $L^{-1}$) and venlafaxine at 37 mg $L^{-1}$ (26 – 48 mg $L^{-1}$). An overview of the $EC_{50}$ values and the respective dose-response curves for non-neonicotinoids are given in annex A (Table A4 and Figure A3, respectively). Overall neonicotinoid insecticides were most acutely toxic to *N. spinipes* whereas only low effects of herbicides and pharmaceuticals were observed. Acute lethality testing of 19 non-neonicotinoid substances with *N. spinipes* resulted in $EC_{50}$ value determination for 4 substances whereas the $EC_{50}$ for the remaining 15 compounds was above the maximum test concentration.

Chemical analysis of the acute lethality testing resulted in constant concentrations (in average ≤ 13 % of concentration reduction after 96h) for all test substances with exception of flufenacet (44 % of reduction after 96h), oxytetracycline (40 % of reduction after 96h) and paracetamol (27 % of reduction after 96h). Detailed information about the chemical analysis is given in annex A (Table A5).

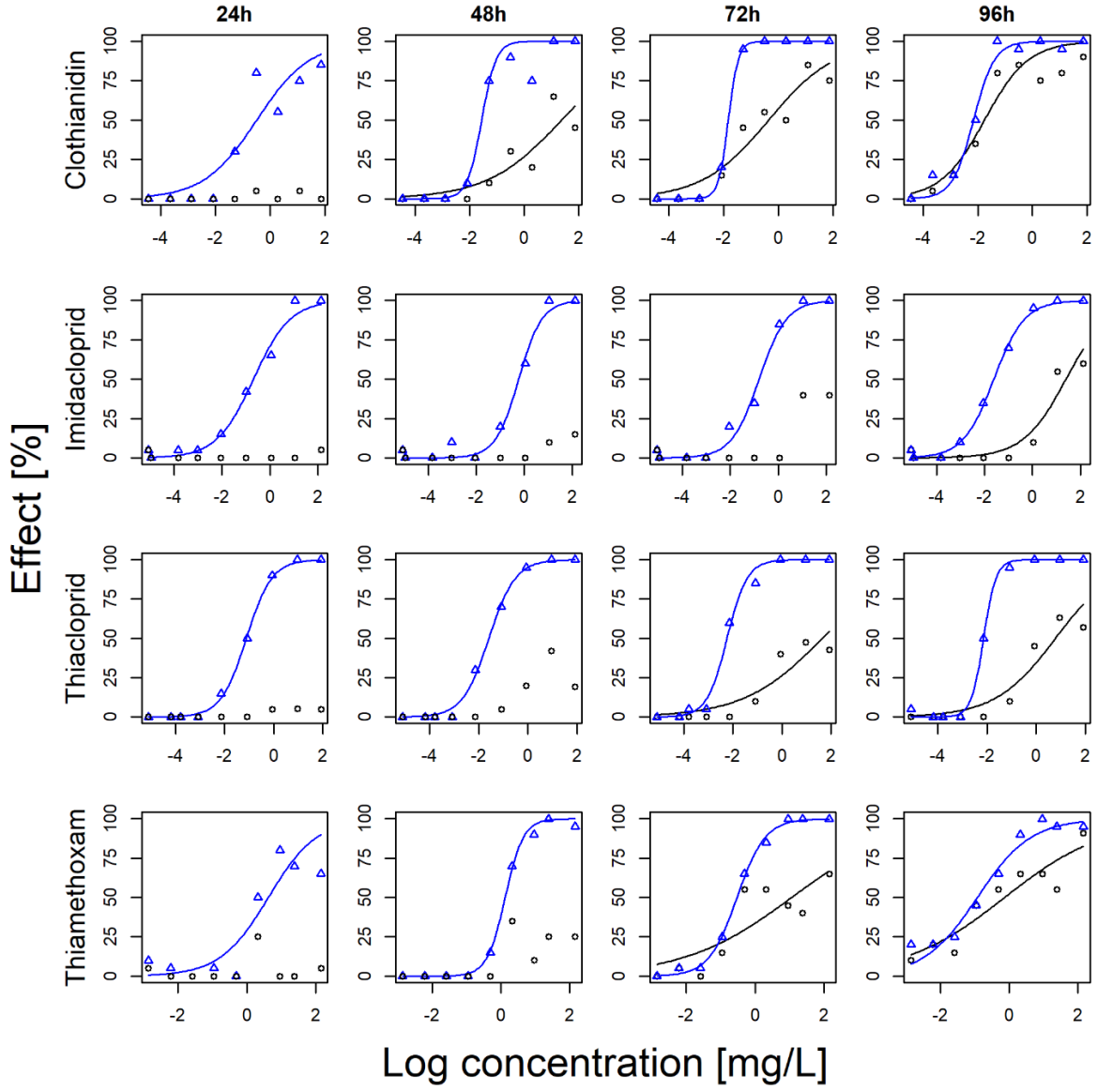



**Figure 4.1** ***Concentration-response curves for*** **Nitocra spinipes** ***exposed to four neonicotinoid insecticides (clothianidin, imidacloprid, thiacloprid and thiamethoxam) measured daily for 96h. Black circles show the mean mortality of quadruplicates in percent, and blue triangles the mean immobilization of quadruplicates in percent. Lines are fitted log-logistic dose-response models.***

**Table 4.2 Acute $EC_{10}$ and $EC_{50}$ (in µg $L^{-1}$) values for the four neonicotinoid insecticides and their 95% confidence intervals (in parentheses) for the two endpoints mortality and immobilization.**

| $EC_{10}$ | Mortality | | | | Immobility | | | |
|---|---|---|---|---|---|---|---|---|
| | **24h** | **48h** | **72h** | **96h** | **24h** | **48h** | **72h** | **96h** |
| Clothianidin | >72,000 | 24 (16 –120) | 0. 94 (0.84 – 3.7) | 0.31 (0.12 – 1.4) | 2.4 (0.9 – 11) | 7.5 (3.8 – 19) | 5.9 (5.8 – 6.0) | 0.99 (0.51 – 2.5) |
| Imidacloprid | >132,000 | >132,000 | >132,000 | 270 (31 – 840) | 4.2 (2.8 – 9.7) | 51 (44 – 108) | 8.8 (5.8 – 21) | 0.96 (0.43 – 1.5) |
| Thiacloprid | >100,000 | 101 (31 – 556) | 13 (5.3 – 57) | 12 (10 – 47) | 5.5 (3.3 – 7.8) | 1.7 (0.78 – 2.6) | 0.72 0.018 – 1.4) | 2.0 (0.52 – 3.4) |
| Thiamethoxam | >142,000 | >142,000 | 4.1 (0.16 – 25) | 0.43 (0.28 – 2.0) | 121 (23 – 597) | 349 (215 – 482) | 38 (24 – 52) | 2.3 (0.81 – 6.1) |
| $EC_{50}$ | Mortality | | | | Immobility | | | |
| | **24h** | **48h** | **72h** | **96h** | **24h** | **48h** | **72h** | **96h** |
| Clothianidin | >72,000 | 24,000 (5,800 – 77,000) | 450 (22 – 1,100) | 18 (6 – 41) | 330 (290 – 940) | 28 (10 – 46) | 15 (15 – 15) | 6.9 (3.2 – 11) |
| Imidacloprid | >132,000 | >132,000 | >132,000 | 25,000 (20,000 – 55,000) | 200 (80 – 330) | 590 (290 – 890) | 160 (70 – 250) | 25 (18 – 31) |
| Thiacloprid | >100,000 | >100,000 | 54,000 (21,000 – 180,000) | 6,100 (2,500 – 16,000) | 76 (62 – 90) | 26 (18 – 330) | 5.7 (3.4 – 81) | 7.2 (6.2 – 8.2) |
| Thiamethoxam | >142,000 | >142,000 | 12,000 (5,900 – 40,000) | 740 (430 – 1,800) | 4,200 (1,700 – 11,000) | 1,300 (1,100 – 1,600) | 300 (250 – 350) | 120 (39 – 200) |

### *4.3.3 Larval development testing with N. spinipes*

***Figure 4.2*** shows concentration-response data and fitted curves for the 7-day larval development testing with *N. spinipes*. The pH varied maximally 0.5 units across all tests and CTs and in average 0.4 units. The salinity varied maximally 0.3 ‰ across all tests and CTs and in average 0.2 ‰. Test concentrations remained constant over 7 days (on average ≤ 11 % variation from test start to test end) with detailed information in annex A (Table A6). 7-day larval development testing of *N. spinipes* resulted in NOECs and $EC_{10}$ (7d) in the low µg $L^{-1}$ range for CLO, IMI and TCP while no effects were observed for TMX up to 99 µg $L^{-1}$. For CLO a significantly higher LDR was observed at 0.08 µg $L^{-1}$ as compared to the control treatment, suggesting a hormetic response. A summary of the endpoints and models used for each compound is given in ***Table 4.3***.

**Table 4.3 Endpoints of the 7-day larval development testing with Nitocra spinipes in µg $L^{-1}$. Shown are the no-observed effect concentration (NOEC), the lowest-observed effect concentration (LOEC), the effect concentration showing 10 % effect ($EC_{10}$) and its 95% confidence interval (95%-CI). Model indicates the model fitted to the data for the determination of the $EC_{10}$. Where the upper limit was fixed to the average larval development ratio of the control treatments ($LDR_{CTL}$).**

| Substance | NOEC | LOEC | $EC_{10}$ | 95%-CI | Model (as in drc package for R)[a] |
|---|---|---|---|---|---|
| Clothianidin | **2.5** | 14 | 2.6[b] | 0.62 – 4.5 | CRS.4c(names = c("b", "d", "e", "f")) |
| Imidacloprid | **4.2** | 13 | 0.18[c] | 0.01 – 2.1 | llogistic2 (fixed = c(NA,0,$LDR_{CTL}$,NA,1)) |
| Thiacloprid | 2.7 | 8.6 | **1.1** | 0.4 – 3.2 | llogistic2 (fixed = c(NA,0,$LDR_{CTL}$,NA,1)) |
| Thiamethoxam | >99 | >99 | >99 | NA[d] | NA[d] |

[a] Concentration response model fitted to the data for the determination of the $EC_{10}$. The upper limit of the larval development ratio (LDR) was fixed to the average LDR in the control treatments ($LDR_{CTL}$) for imidacloprid and thiacloprid. CRS.4c is a hormesis model (Cedergreen et al. 2005); llogistic2 is a log-logistic model.

[b] Uncertain model fit and $EC_{10}$ value because only one tested concentration showed significant negative effect

[c] Uncertain $EC_{10}$ value because extrapolated below lowest test concentration

[d] Not Applicable

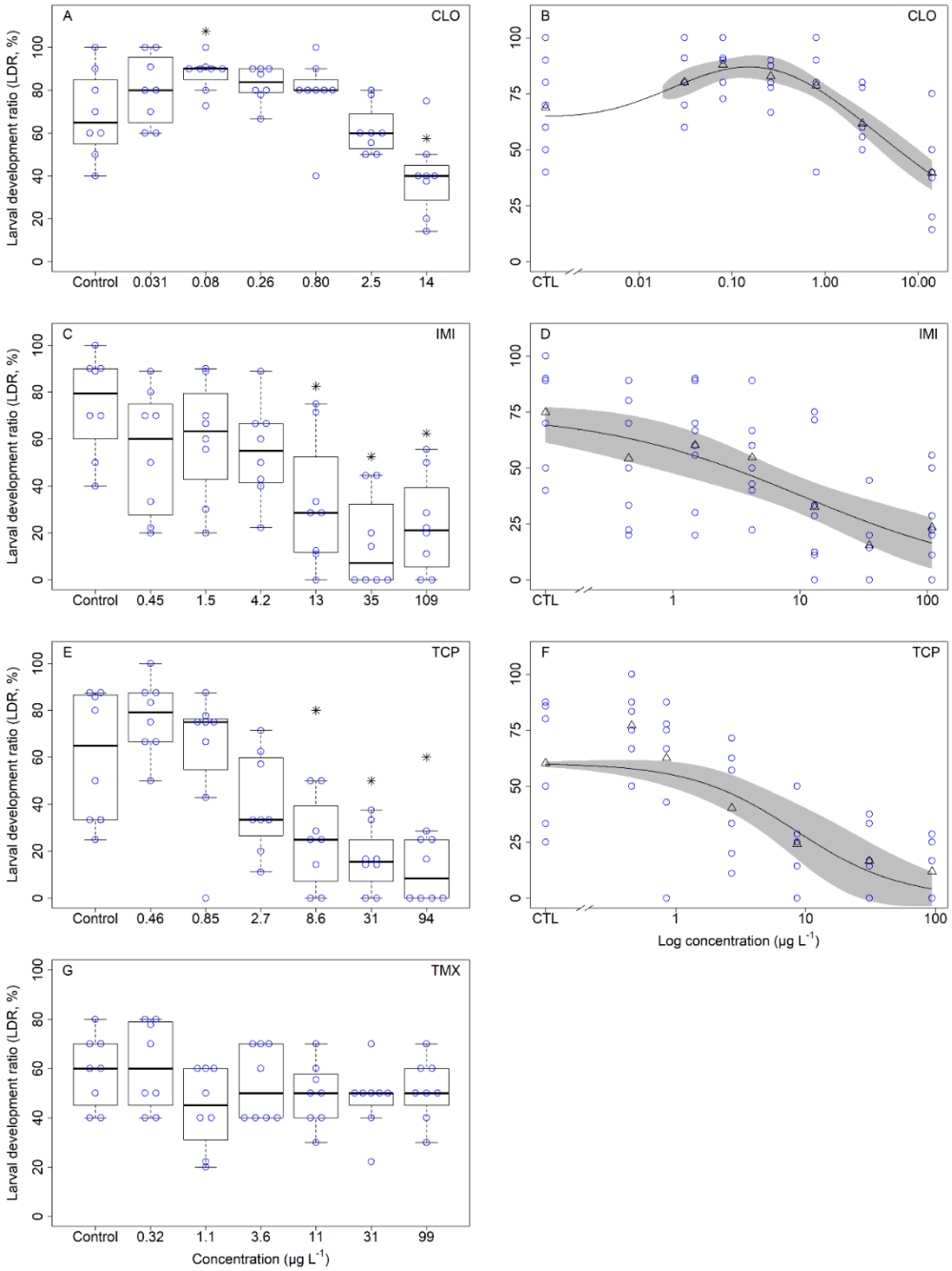



**Figure 4.2 Results of the 7-day larval development tests with *N. spinipes* for clothianidin (CLO, A and B), imidacloprid (IMI, C and D), thiacloprid (TCP, E and F) and thiamethoxam (TMX, G). The boxplots (left column) show the results after 6 (CLO and TMX) or 7 (IMI and TCP) days of exposure for the different concentration treatments as compared to the control treatments (Control). The boxes indicate the 25$^{th}$ to 75$^{th}$ percentiles and the upper and lower limits indicate the minimum and maximum data points excluding outliers (more and less than 1.5x upper and lower quartile). The bold line shows the median larval development ratio (LDR). Treatments marked with an * indicate concentrations with a statistically significant difference (Mann-Whitney-U test, p-value < 0.05) of LDR compared to the control treatment. Blue circles in both plots represent the individual data points per replicate. The concentration response curves (right column) show the fitted dose response models for the LDR vs. the logarithmic concentration in µg L$^{-1}$. The black triangles indicate the average LDR per concentration treatment and the grey zone indicates the 95 % confidence interval on responses predicted by the dose-response model.**

### *4.3.4 Deriving Environmental Quality Standards*

#### *4.3.4.1 Conventional approach*

An overview of all derived EQS values can be found in **Table 4.4**. A summary of the available toxicity data from the two databases used for the derivation of EQS values for all four neonicotinoid insecticides, including the used AFs, is shown in **Table 4.5**.

In the TGD for deriving EQS, “data for additional marine taxonomic groups” has been defined as “data from studies with marine organisms other than algae, crustacean and fish, and/or having a life form or feeding strategy different from that of algae, crustaceans or fish” [6]. This definition gives a certain degree of freedom to the risk assessor and makes an EQS derivation a somewhat subjective process that needs expert judgement and justification. Assumptions and justifications taken during this exercise are provided in annex A (section A2.4).

A link to a document with an overview of the complete data used for the EQS derivation of each substance can be found in annex A. *N. spinipes* was found to be the most sensitive species for long-term exposure to CLO. Further, for the derivation of the saltwater AA-EQS (AA-$EQS_{sw}$) of TMX, the availability of long-term data for *N. spinipes* as an additional marine taxonomic group led to the reduction of the AF from 100 to 50. The lowest Maximum Allowable Concentration (MAC)-EQS and AA-EQS were derived for IMI with 0.065 µg $L^{-1}$ and 0.002 µg $L^{-1}$, respectively. The highest difference between MAC-EQS and AA-EQS was observed for TMX with the latter being 325 times lower.

**Table 4.4 Derived Environmental Quality Standards (EQS) for four neonicotinoid insecticides (by dividing the lowest toxicity value by the assessment factor as reported in Table 3). Derived are the Maximum Allowable Concentration (MAC-) EQS and the Annual Average (AA-) EQS for both fresh water (fw) and salt water (sw) environments. All values are expressed in µg $L^{-1}$.**

| Substance | MAC-$EQS_{fw}$ | MAC-$EQS_{sw}$ | AA-$EQS_{fw}$ | AA-$EQS_{sw}$ |
|---|---|---|---|---|
| Clothianidin | 0.23 | 0.23 | 0.25 | 0.05 |
| Imidacloprid | 0.065 | 0.065 | 0.01 | 0.002 |
| Thiacloprid | 0.46 | 0.46 | 0.024 | 0.0048 |
| Thiamethoxam | 5.2 | 5.2 | 0.081 | 0.016 |

Table 3 Data used for saltwater (sw) Environmental Quality Standard (EQS) derivation for neonicotinoid insecticides. All effect values are given in µg $L^{-1}$.

**Table 4.5 Data used for saltwater (sw) Environmental Quality Standard (EQS) derivation for neonicotinoid insecticides. All effect values are given in µg $L^{-1}$.**

| Substance | MAC-$EQS_{sw}$ Lowest $EC_{50}$ | Endpoint | Species | Test duration [d] | Total number of species | AF | AA-$EQS_{sw}$ Lowest NOEC or $EC_{10}$ | Endpoint | Species | Test duration [d] | Total number of species | AF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clothianidin | 2.3 | Mortality | *Chironomus dilutus*[a] | 4 | 10 | 10 | 2.5 | Larval development | *Nitocra spinipes*[b,c] | 6 | 8 | $10^{fw}$ $50^{sw}$ |
| Imidacloprid | 0.65 | Mortality | *Epeorus longimanus*[a] | 4 | 13 | 10 | 0.1 | Length | *Epeorus sp.*[a] | 20 | 8 | $10^{fw}$ $50^{sw}$ |
| Thiacloprid | 4.6 | Mortality | *Baetis rhodani*[a] | 4 | 9 | 10 | 0.24 | Mortality | *Cloeon dipterum*[a] | 7 | 7 | $10^{fw}$ $50^{sw}$ |
| Thiamethoxam | 52 | Mortality | *Cloeon dipterum*[a] | 4 | 9 | 10 | 0.81 | Mortality | *Cloeon dipterum*[a] | 28 | 6 | $10^{fw}$ $50^{sw}$ |

[a]insects
[b]copepods
[c]this study
[fw/sw]freshwater/saltwater

#### 4.3.4.2 *Using acute $EC_{10}$ values for AA-EQS derivation*

We found the acute $EC_{10}$ (96h) values of CLO, IMI and TMX for adult *N. spinipes* to be lower than their chronic $EC_{10}$ (7d) values for larvae (***Table 4.2*** and ***Table 4.3***). Since the protection goal for a species includes all life stages and endpoints, we also calculated AA-EQS for the scenario in which we considered the acute $EC_{10}$ (96h) values for *N. spinipes*. This had an impact on the derived AA-EQS for CLO and TMX, which became a factor 8 and 2 lower, respectively, compared to the regulatory conventional method (only using chronic data), resulting in 0.0062 and 0.0086 µg $L^{-1}$ for CLO and TMX, respectively. The AA-EQS for IMI and TCP remained unchanged.

### 4.3.5 *Risk characterization*

#### 4.3.5.1 *Conventional EQS derivation*

***Figure 4.3*** gives an overview of the calculated TUs for SC2 – SC5. Overall, the risk characterization based on TUs for neonicotinoid insecticides showed that most risks were observed for IMI and the harbour of Ostend (***Figure 4.3***). The $TU_{mix}$ was mainly driven by IMI and to a minor extent by TCP or TMX. Specific mixture risks (i.e. $TU_{mix}$ > 1 when no single neonicotinoid by itself had TU>1) were not observed with one exception, i.e. the passive sample from HZ in SC2. In general, we observed TUs > 1 only with grab sample-based data, with the exception for SC2. The exceedance of PNECs at two harbor sites suggests the presence of ecological risks due to neonicotinoids in the harbors of the BPNS. The $TU_{mix}$ at the coastal locations consistently being ≥ 0.1 indicate a relatively limited MoS for neonicotinoid risks in the BPNS.

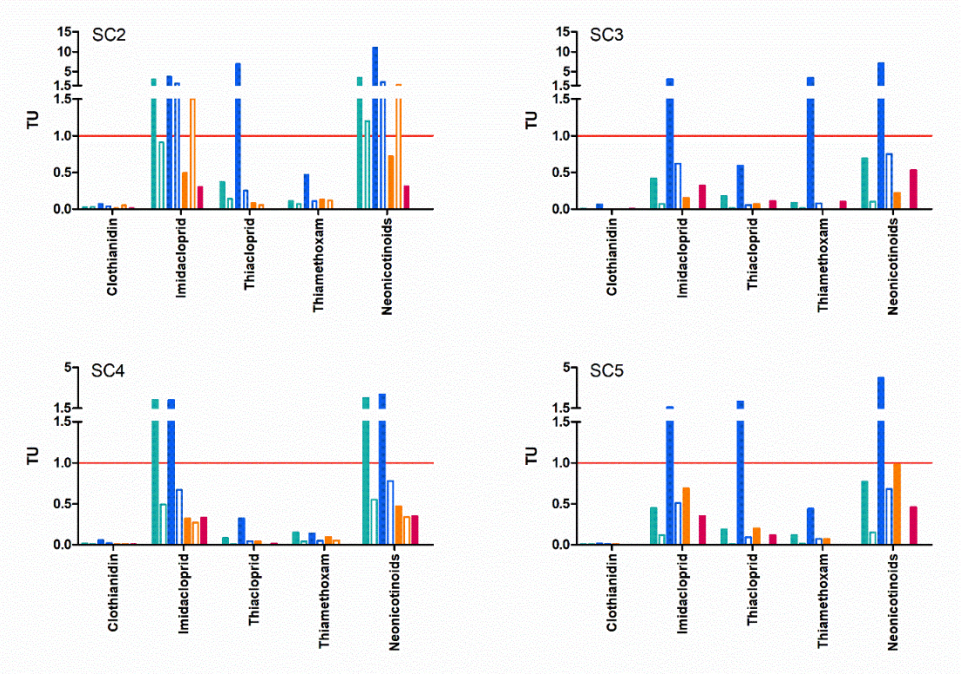



**Figure 4.3** ***Risk quotients (RQ) for sampling campaigns (SC) 2-5 in the Belgian part of the North Sea based on grab sampling (filled bars) and passive sampling data (empty bars) and derived PNECs. Shown are the toxic units (TU) for four neonicotinoid insecticides and their mixture (Neonicotinoids) for the four sampling locations HZ (green), HO (blue), OZ_MOW1 (orange) and OO_X (pink). Bars show the TU calculated based on the average measured concentration.***

#### 4.3.5.2 Using acute $EC_{10}$ values for EQS derivation

When basing the risk characterization on the EQS derived including acute $EC_{10}$ values that were lower than chronic $EC_{10}$ values for *N. spinipes*, the overall risk characterization across SC2 – SC5 did not change considerably. The only exception was the exceedance of RQ = 1 of the $TU_{mix}$ at SC5 MOW1.

## 4.4 Discussion

In acute lethality testing with *N. spinipes,* neonicotinoid insecticides were found to be the most toxic among 23 PCPs, pesticides and pharmaceuticals that had been detected in SC1. Based on the results of these tests long-term effects of neonicotinoid insecticides were further investigated in 7-day larval development tests with *N. spinipes*. Ultimately, EQS were derived and risks for the BPNS were assessed for four neonicotinoids individually and as a mixture. During the acute toxicity testing, we found that for neonicotinoid insecticides immobilization was a more sensitive endpoint than mortality. Finally, the risk characterization revealed that the $TU_{mix}$ was mainly driven by IMI and to a minor extent by TCP or TMX. Specific mixture risks (i.e. $TU_{mix}$ > 1 when no single neonicotinoid by itself had TU>1) were not observed with one exception, i.e. the passive sample from HZ in SC2. In general, we observed TUs > 1 only with grab sample-based data, with the exception for SC2. The exceedance of PNECs at two harbor sites suggests the presence of ecological risks due to neonicotinoids in the harbors of the BPNS. The $TU_{mix}$ at the coastal locations consistently being ≥ 0.1 indicate a relatively limited MoS for neonicotinoid risks in the BPNS.

### 4.4.1 Growth inhibition testing with P. tricornutum

The data from 72h growth inhibition testing suggests a low sensitivity of the diatom *P. tricornutum* to all tested substances. The $EC_{50}$ values (72h) for atenolol, bezafibrate, and carbamazepine were in close correspondence with those reported by Claessens et al. (2013).

### 4.4.2 Acute lethality testing with N. spinipes

For substances other than the neonicotinoid insecticides, the lowest $LC_{50}$ (96h) was observed for amantadine with 4.8 mg $L^{-1}$. This indicates that these substances are of low toxicity to *N. spinipes*.

Acute toxicity testing of neonicotinoid insecticides with *N. spinipes* revealed that immobilization was a more sensitive endpoint as compared to mortality. Similar effects have been observed for IMI on 3 freshwater ostracods and 2 freshwater cladoceran species [85] and for TCP on a freshwater copepod species [86]. Sanchez-Bayo and Goka (2006) suggested that immobilization due to neonicotinoid exposure can seriously endanger populations of these organisms in the wild and listed the following 2 main reasons: (1) immobilization makes the zooplankton easy prey vulnerable to attacks by their numerous predators, (2) the paralysis induced by neonicotinoids is likely to cause starvation for predators because they experience reduced mobility which might result in a lower predation success [87]. Overall, neonicotinoids elicited acute toxic responses from *N. spinipes* over a concentration range of 17-fold, with CLO being the most toxic and TMX being the least toxic indicating that toxicity among neonicotinoids can vary widely. The same has been confirmed by Raby et al. (2018) for the freshwater

crustacean *Hyalella azteca* with $EC_{50}$ values ranging 81-fold [88]. Differences in toxicity were also found upon topical treatment of honey bees (*Apis mellifera*) with nitro-containing neonicotinoids (including CLO, IMI and TMX) being more toxic than cyano-group containing ones (including TCP) [89]. The lower toxicity of cyano-group containing neonicotinoids was associated with their faster biotransformation and the existence of different nicotinic acetylcholine receptor subtypes [90].
While $EC_{50}$ values are a commonly recognized endpoint for acute toxicity studies, barely any attention is given to acute $EC_{10}$ values, even though a 10% acute effect may also result in a population decline. The here derived immobilization $EC_{10}$ (96h) values for *N. spinipes* (Table 1) were a factor of 7.0, 26, 3.6 and 52 lower than the respective $EC_{50}$ (96h) values for CLO, IMI, TCP and TMX, respectively.
Our study has shown that immobilization of *N. spinipes* exposed to neonicotinoid insecticides is likely to occur at concentration levels in the low µg $L^{-1}$-range and such immobilization might negatively affect *N. spinipes* populations. Further, the four neonicotinoids could be ranked according to their acute toxicity as follows: CLO > TCP >> IMI >> TMX.

### *4.4.3 Larval development testing with N. spinipes*

7-day larval development testing of *N. spinipes* resulted in NOECs in the low µg $L^{-1}$ range for CLO, IMI and TCP while no effects were observed for TMX up to 99 µg $L^{-1}$. These findings are comparable to chronic $LC_{10}$ (7d) values for the freshwater crustacean *H. azteca* ranging from 2.8 µg $L^{-1}$ (CLO) to 160 µg $L^{-1}$ (TMX) [91]. Further, the 7-day larval development results confirmed the neonicotinoid potency ranking (CLO > TCP > IMI > TMX) suggested by acute testing and in a study investigating acute (7d) and chronic (28d) effects of the same neonicotinoids on the freshwater amphipod *H azteca* [91]. Thus, despite similar structure and the same mode of action, neonicotinoid insecticides differ in their toxicity. These differences are most likely related to variability in their toxicokinetics and/or toxicodynamics [92] determined by e.g. differences in binding sites, binding affinities and/or specificity of binding between compounds [93-96].
Next to that, NOEC and $EC_{10}$ values were observed to be within a factor of 2.5 for CLO, TCP and TMX, while a factor of 23 was observed for IMI. This difference for IMI did not have any influence on the EQS-derivation, but requires careful evaluation when using these data in risk assessment. The $EC_{10}$ for IMI was extrapolated below the lowest test concentration, which may explain the relatively high uncertainty on the $EC_{10}$ (CI = 0.01 – 2.1 µg $L^{-1}$). Thus, for IMI the use of the NOEC is recommended over using the $EC_{10}$.

### *4.4.4 Deriving Environmental Quality Standards*

When deriving EQS, one faces several challenges and needs to consider many different aspects. In the following paragraph we would like to list a few of these challenges, explain how we dealt with them and justify our decisions. In addition, information about data reliability and detailed justifications for certain choices can be consulted in annex A.
Several authors described midges and mayflies [97-99] as the most sensitive aquatic organisms to neonicotinoids in both acute and chronic exposure scenarios. While literature data confirmed this generally, our experiments with *N. spinipes* resulted in lower $EC_{10}$ values (7-day larval development) for CLO as compared to the lowest NOEC/$EC_{10}$ values in the used databases.

#### 4.4.4.1 Ecotoxicity databases

EQS derivation is a hazard-based approach aiming to define thresholds with a high protection goal for the freshwater and marine environment. It is therefore crucial to reduce any uncertainty to a minimum by including as much data as possible into the decision-making process [6]. Here, we focused on the US EPA ECOTOX and OPP databases because they complemented each other due to (mainly) different data sources, and could be regarded as to cover the majority of toxicity data directly available to us at the time of retrieval. While online databases provide an extensive amount of toxicity data for neonicotinoids, there is a clear lack of data for marine species. Next to that, data reliability is a critical point and requires thorough checking which might lead to a reduction of the already scarce data for e.g. EQS derivation.

#### 4.4.4.2 The use of freshwater and saltwater species data

The use of both freshwater and saltwater data led to an overall increase of data resulting in a decrease of uncertainty for the EQS derivation. Nonetheless, merging the two datasets for the EQS derivation of neonicotinoids can be questioned due to the fact that freshwater insects have been shown to be among the most sensitive species to neonicotinoids [97, 99]. These insects usually spend their juvenile stages in freshwater habitats until maturation, but there exist no insects with a similar life form in saltwater. Only a very limited number of insect species have shown tolerance to low salinity [100]. Thus, the relevance of insect data for the derivation of $EQS_{sw}$ is questionable. On the other hand, data for marine species in risk assessment or EQS derivation is usually scarce and often, basing $EQS_{sw}$ derivation on a combination of freshwater and saltwater data is the best practice to lower the AFs in use [7].
The freshwater : saltwater data ratio in our datasets was 47:12, 175:8, 73:8 and 63:7 for CLO, IMI, TCP and TXM, respectively. Thus, data for saltwater species represented only 4 – 20 % of the available data for EQS derivation. Excluding freshwater data in this case would thus have led to an increase of the AFs from 10 to 50 for IMI, TCP and TMX but no change for CLO for the MAC-EQS. For the AA-EQS it would have led to an increase from 50 to 100 for CLO, from 50 to 500 for IMI and TCP, and from 50 to 1000 for TMX. This would result in a slight increase of the MAC-EQS for CLO and IMI (to 0.32 and 0.025 µg $L^{-1}$, respectively) and an overall considerable decrease of the MAC-EQS for TCP and TMX (to 0.20 and 2.4 µg $L^{-1}$, respectively) and a decrease of the AA-EQS for CLO, TCP and TMX (to 0.025, 0.00033 and 0.0022 µg $L^{-1}$, respectively). Finally, the derivation of an AA-EQS for TMX would not have been possible since no endpoint would have been available.

#### 4.4.4.3 Extrapolation using assessment factors

One important difference between freshwater and saltwater EQS derivation is the use of different AFs. AFs used for the saltwater environment are usually set a factor of 10 higher to deal with the higher biodiversity in the marine environment and the ongoing uncertainty to represent the most sensitive organisms [6]. Next to the basic set of toxicity data (algae, crustacean and fish), the AF for saltwater EQS derivation can be further reduced when data for additional marine species is available. This includes taxa different from the basic set of algae, crustacean and fish such as e.g. mollusks or echinoderms, but also marine organisms belonging to the taxa algae, crustacean or fish with either a different life form or feeding strategy [6]. In the present study we used toxicity data of several freshwater and saltwater algae and the aquatic plant *Lemna gibba*. If data was available for several algae, they were

always considered as representatives of one taxonomic group and the lowest endpoint was considered for EQS derivation. *L. gibba* was considered as an additional freshwater species representing a separate taxon. For crustaceans, overall data was available for 8 different species representing 6 order, i.e. amphipoda, cladocera, decapoda, isopoda, mysida and harpacticoida. Short-term data for an additional marine taxonomic group was available in the form of mollusk data for all four neonicotinoids. Long-term data was available for *A. bahia* and additionally provided by our 7-day larval development tests with *N. spinipes* for all substances but TMX. AFs in use ranged from 10 to 50 proving a relatively low uncertainty for the EQS derivation.

#### *4.4.4.4 Using acute $EC_{10}$ values for EQS derivation*

In this study, I observed a rather rare case where short-term exposure of adult organisms resulted in lower effect concentrations (mortality or immobilization 96h-$EC_{10}$ values) as compared to long-term exposure of their early life stages (7d-NOECs). It is commonly recognized that early-life stages are usually more sensitive than adult organisms of the same species, but exceptions do exist. Holan et al. (2018) found adult individuals of the marine bivalve *Gaimardia trapesina* to be more sensitive than juveniles when exposed to copper [101]. Since one of the overarching goals of the derivation of EQS is to protect species at a population level, there is no clear reason for not including acute $EC_{10}$ values in the derivation of the AA-EQS. The consideration of $EC_{10}$ (96h) values from *N. spinipes* short-term exposure to neonicotinoid insecticides for the AA-EQS derivation led to a more conservative AA-EQS for CLO and TMX. These findings highlight the importance of allowing some flexibility when deriving EQS. Here, we show that using short-term $EC_{10}$ values as additional endpoints for the AA-EQS derivation may lead to a more adequate protection of *N. spinipes* populations.

#### *4.4.4.5 Comparison of EQS and literature threshold values*

Due to their extensive use and subsequent detection in the aquatic environment [98], neonicotinoid insecticides have been studied a lot using a manifold of test setups and species [99]. They have been found to adversely affect a wide range of non-target organisms, specifically insects [99]. Nevertheless, two (IMI, TCP) out of four neonicotinoids tested in our study currently remain approved for the European market, with CLO and TMX being banned with national exceptions for a variety of countries. Notably, the use of IMI is restricted to application in permanent greenhouses [102].

In a review about neonicotinoid insecticides in the Canadian aquatic environment, Anderson et al. (2015) concluded that in terms of toxicity data most studies have been performed for IMI, while data for CLO and TMX was generally scarce [99]. We do not fully agree with this statement since – based on data derived from only two of the many existing ecotoxicity databases – we found toxicity data for 16 freshwater species covering 6 different taxonomic groups and for 5 saltwater species covering 4 taxonomic groups for CLO which, combined, formed a solid basis for EQS derivation. For TCP and TMX, data for saltwater species was indeed very scarce and EQS derivation for the saltwater environment was associated with a higher degree of uncertainty.

The MAC-EQS derived in our study were a factor of 1.7 to 48 lower than threshold values reported in literature so far (annex A, Table A7). Whereas the MAC-EQS for IMI and TMX were only slightly lower (1.7 to 6 times lower), the MAC-EQS for CLO and TCP were up to a factor

of 48 and 41 lower than the US-reported aquatic life benchmark (LB) for invertebrates [103]. Nevertheless, the derivation of EQS and LB differs significantly with the latter being based on either the lowest 48h- or 96h-$EC_{50}$ or $LC_{50}$ of a standardized test with usually a midge, a scud or a daphnid. This $EC_{50}$ is then reduced using a level of concern (LOC, comparable to an AF) of 0.5 for the acute value. For LBs based on the lowest no-observed adverse effect concentration (NOAEC) from a life-cycle test with usually a midge, a scud or a daphnid, a LOC of 1 is applied to the chronic endpoint. Thus, an LB only takes data from the respective taxon (in this case crustacean data) into account and is as such not really comparable to an EQS that aims to protect a whole ecosystem rather than few taxonomic groups. In a Dutch study, Smit et al. (2015) derived a MAC-$EQS_{fw}$ of 0.065 µg $L^{-1}$ for IMI using the AF approach, resulting in the exact same value than in our study and thereby confirming our approach [104].
The derived AA-EQS, on the other hand, were within a factor of 0.006 – 202 of threshold values reported in literature so far. There was a relatively high discrepancy between the derived AA-EQS and the US-LB [103] or the Canadian long-term thresholds [105] due to very distinct derivation methods. Comparison with PNEC values proposed by the Joint Research Center [72] on the other hand, resulted in the AA-EQS being a factor of 0.42 – 4.2 of those reported in literature. This is logic due to the very similar approaches for derivation of EQS and PNEC values under European legislation.

### *4.4.5 Risk characterization*

Overall, risk characterization resulted in rather comparable patterns for SC3 – SC5 whereas SC2 resulted in more exceedances of RQ = 1 including the only exceedance at a coastal location. Neonicotinoid insecticides have been identified as contaminants of concern for aquatic ecosystems due to their frequent occurrence and relatively low effect thresholds in various organism groups (e.g. insects and crustaceans) [85, 97]. Mixtures of neonicotinoids (including the here investigated ones) have been reported to represent a significant threat to 14/19 surface waters, and exceedance of individual MAC-$EQS_{fw}$ in another 22/27 monitoring studies found in literature was reported [97]. In addition, maximum concentrations of IMI measured in the Llobregat River (north-east Spain) have been reported to be close to the short-term threshold (0.1 - 0.07 µg $L^{-1}$) and exceeding the long-term threshold (0.03 µg $L^{-1}$) proposed in this study [106]. For TMX risks due to short-term exposure are very unlikely to occur even in freshwater ecosystems due to the relatively high MAC-EQS of 5.2 µg $L^{-1}$ which has been concluded before [107]. The proposed AA-EQS on the other hand was found to be exceeded at HO and long-term exposure to such concentrations may pose a risk to the Belgian marine environment. Further, we found exceedance of the $TU_{mix}$ for the four neonicotinoids at HZ where individual substances did not exceed the threshold. While risk assessment for individual neonicotinoids and their mixtures has been conducted for a variety of freshwater ecosystems, our study is to our knowledge the first to evaluate potential risks for marine ecosystems. The exceedance of the AA-EQS at the two investigated harbors should serve as an early warning for the BPNS. This is further supported by the relatively high AA-EQS-based average $RQ_{mix}$ of 0.60 and 0.41 observed across all SC for OZ_MOW1 and OO_X resulting in a relatively limited MoS for this sampling location. In addition, the ban of CLO and TMX in Europe and the currently restricted use of IMI might lead to an increased use of TCP as an alternative neonicotinoid insecticide, resulting in an increasing input of this substance into marine waters. This is disconcerting since TCP was among the two substances contributing

the most to the ∑TU together with IMI. Thus, a replacement of banned neonicotinoid insecticides by other neonicotinoids might turn out to be a very regrettable solution.

## 4.5 Conclusion

Neonicotinoid insecticides are used worldwide and have become of global concern for the aquatic environment. Harpacticoid copepods are, unlike many other crustaceans, very sensitive to neonicotinoids and have been shown to be of outmost importance for the community in the BPNS where they comprise 66 % of the total zooplankton abundance [75]. Acute toxicity testing with *N. spinipes* revealed that immobilization is a much more sensitive endpoint than the standard endpoint mortality (especially in the first 48h). Overall, the data generated for *N. spinipes* led to a refinement of the saltwater AA-EQS for CLO, and contributed considerably to the reduction of uncertainty (AF) in the definition of the saltwater AA-EQS for TMX. In addition, short-term $EC_{10}$ values for AA-EQS derivations resulted in a reduced AA-EQS for CLO and TMX, thus highlighting the importance of short-term $EC_{10}$ values for threshold values (e.g. EQS) derived for a protection on the population level. Compared to measured concentrations in the BPNS, we found exceedance of the AA-EQS for all neonicotinoids but CLO across the different SCs. In general, there was no clear pattern visible but IMI showed the highest overall exceedance of the four neonicotinoids. Finally, the mixture RQ ($RQ_{mix}$) was above 1 at HO for all four SCs, at HZ for SC2 and SC4 and at OZ_MOW1 for SC2. In general, we found a relatively low MoS for the coastal sampling locations in the BPNS. Given the key role of copepods for the total zooplankton abundance in the BPNS and considering their high sensitivity, potential risks of neonicotinoids for the BPNS in the future cannot be excluded and further monitoring is strongly advised.

The ecotoxicity testing of a selected number of CECs identified especially neonicotinoid insecticides to be of concern for the BPNS. When deriving EQS for the four neonicotinoids we found many obstacles and ambiguities that require in-depth ecotoxicological knowledge and expert judgement. This thesis aimed to investigate and prioritize CECs for the BPNS from an ecotoxicological point of view. Considering the broad spectrum of chemical substances that have been detected and quantified alone in the BPNS [17, 71, 108, 109] it rapidly becomes clear that deriving marine EQS or PNEC values for all substances one by one is a gargantuan task. From a regulatory perspective, there are only three promising approaches to handle this complex task: i) automation of EQS/PNEC derivation and associated risk assessment on a substance-by-substance level ii) effect-directed analysis to reduce sample complexity and identify those chemicals that might cause adverse effects or iii) moving from a single substance-based to a mixture-based risk assessment. In this thesis, the first and third concepts are addressed. In chapter 5 we provide a first step towards an individual substance-based automated (marine) screening-level risk assessment while in chapter 7 an effect-based monitoring approach allowing a MoS determination based on ecotoxicity testing of ERCMs was developed and applied to the BPNS.

# 5

# Development and application of an automated approach for screening-level risk assessment of chemicals of emerging concern

Redrafted from:

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# 5 Development and application of an automated approach for screening-level risk assessment of chemicals of emerging concern

## 5.1 Introduction

The monitoring of marine waters under the Marine Strategy Framework Directive (MSFD) with the goal of safeguarding environmental water quality in terms of chemical pollution so far has focused on the evaluation of a few selected compounds [22]. For this purpose, concentrations of these priority pollutants are analytically determined in different compartments (i.e. water, sediment and biota) and compared against Environmental Quality Standards (EQS) [4]. Highly sensitive and reliable analytical methods are needed for the analysis of the very diverse substances occurring in marine waters. Shifting from tandem mass spectrometry (MS/MS) to high-resolution mass spectrometry (HRMS) and the development of passive sampling have increased the number of compounds that can be analyzed simultaneously in aquatic matrices to virtually unlimited [110].

Similar to increasing information from environmental monitoring data, growing legislative requirements have laid the foundation for the generation of more standardized ecotoxicity datasets for a broad range of chemicals [111]. REACH for example triggered the creation of unprecedented amounts of ecotoxicity data and guarantees continuous efforts for existing and novel substances [12]. Lots of this data is publicly available via the European Chemicals Agency [112] or comparable databases such as e.g. the US EPA ECOTOXicology Knowledgebase [80]. In an attempt to copy the well-established concept of the threshold of toxicological concern (TTC) for assessing human safety of chemicals related to food safety, Gutsell et al. (2014) established the ecological TCC (eco-TCC) for consumer product chemicals [113]. TTC approaches have benefits for screening-level risk assessments, including the potential for rapid decision-making [114]. Yet, the TTC concept requires categorization of chemicals with respect to mode of action, chemical functional use or chemical category and classification can be a complex decision. In addition, a hazard decision associated with each chemical and test result is required and could be based on existing thresholds (e.g. PNEC) or results from a particular test species [114].

In a first implementation attempt, Connors et al. (2019) developed a curated aquatic toxicology database, EnviroTox, for the initial purpose of developing eco-TTCs as screening tools for chemical hazard assessment. The EnviroTox platform features 3 analysis tools: i) a PNEC calculator, ii) an eco-TCC distribution tool, and iii) a chemical toxicity distribution tool. Inspired by the EnviroTox database, in this chapter we developed an automated calculation algorithm and programmed it in R to derive screening-level marine PNECs for CECs based on a database export from the US EPA ECOTOX Knowledgebase [80]. In addition, we extended the applicability of the calculation algorithm to calculate RQs for all target substances of the NewSTHEPS project based on environmental monitoring data from the BPNS. The developed automated screening level risk assessment was used to i) compare spatio-temporal distributions of risk quotients (RQs) for 4 sampling locations in the BPNS, ii) explore potential differences between a water grab sample-based and a passive sampler-based screening-level risk assessment and iii) help prioritizing specific chemicals or chemical classes for further research.

## 5.2 Materials and methods

Ecotoxicity data was downloaded from the US EPA ECOTOX Knowledgebase [80] on August 6th, 2019. Search criteria were the following:

1. Chemicals: CAS number of the target substances (see annex A Table A1).
2. Effect Measurements: Growth, mortality, population and reproduction groups.
3. Endpoints: AC xx, LC/LD xx, EC/ED xx, IC/ID xx, NOEC and NOEL
4. Species: All
5. Test conditions:
    a. Test Locations: Lab
    b. Exposure Media: Fresh water and salt water
    c. Exposure Types: All
    d. Control Types: All
    e. Chemical Analysis: All

All other settings were left at default and data downloaded as .xlsx file. After retrieval, data was converted into a .csv file for further use.

### *5.2.1 Data processing*

#### *5.2.1.1 Data filtering*

In order to remove endpoints that are not conform with those useable for PNEC calculations, we removed all data with effect levels other than 10 % and 50 % concentrations/doses (e.g. other than $EC_{10}$ and $EC_{50}$ values) or other than NOEC and NOEL values. Next, endpoints expressed in non-mass-based units (i.e. other than "AI mg $L^{-1}$", where AI = active ingredient) were removed from the dataset. Finally, data with an exposure time < 2 day(s), day(s) post-hatch, day(s) post swim up or day(s) post-fertilization were removed from the dataset.

#### *5.2.1.2 Species group classification*

All data was classified into following species groups by adding an extra column to the database (nomenclature between quotation marks refer to classifications used in the US EPA Knowledgebase):

- algae (including "Algae" and "Algae; Standard Test Species")
- amphibians ("Amphibians", "Amphibians; Standard Test Species" and "Amphibians; Standard Test Species; U.S. Exotic/Nuisance Species")
- corals ("Boulder Star Coral", "Coral", "Great Star Coral", "Mustard Hill Coral", "Smooth Cauliflower Coral", "Staghorn Coral", "Stony Coal" and "Thin Finger Coral")
- crustaceans ("Crustaceans", "Crustaceans; Standard Test Species", "Crustaceans; Standard Test Species; U.S. Exotic/Nuisance Species" and "Crustaceans; U.S. Exotic/Nuisance Species")
- fish ("Fish", "Fish; Standard Test Species", "Fish; Standard Test Species; U.S. Exotic/Nuisance Species", "Fish; Standard Test Species; U.S. Threatened and Endangered Species", "Fish; U.S. Exotic/Nuisance Species" and "Fish; U.S. Threatened and Endangered Species")

- insects (“Insects/Spiders”, “Insects/Spiders; Standard Test Species” and “Insects/Spiders; U.S. Exotic/Nuisance Species”)
- molluscs (“Molluscs”, “Molluscs; Standard Test Species”, “Molluscs; Standard Test Species; U.S. Exotic/Nuisance Species” and “Molluscs; U.S. Exotic/Nuisance Species”)
- plants (“Flowers, Trees, Shrubs, Ferns”, “Flowers, Trees, Shrubs, Ferns; Standard Test Species”, “Flowers, Trees, Shrubs, Ferns; U.S. Exotic/Nuisance Species” and “Flowers, Trees, Shrubs, Ferns; Standard Test Species; U.S. Exotic/Nuisance Species”)
- rotifers (“Rotifer” and “Rotifer Phylum”)
- sea urchins (“Purple Sea Urchin”, “Purple-Spined Sea Urchin”, “Sand Dollar”, “Sea Urchin” and “Sea urchin, Echinoderms”)
- worms (“Worms” and “Worms; Standard Test Species”)

The few available data for fungi and protozoa were removed since these species are not typically used for aquatic risk assessment.

### *5.2.2 Definitions*

Environmental risk assessment requires a clear separation between short-term and long-term endpoints. To classify the database entries into these two categories, endpoints were classified in one of these two categories based on endpoint type and the exposure duration, which was specifically defined for each species group.

Short-term data was defined differently for primary producers (algae and plants) than for all other species groups. Only “$AC_{50}$”, “$EC_{50}$”, “$IC_{50}$”, “$LC_{50}$”, “$AD_{50}$”, “$ED_{50}$”, “$ID_{50}$” or “$LD_{50}$” values (i.e. only 50% effect levels) with test durations of ≤ 7d (for primary producers) and ≤ 4d (for all other species) were considered as short-term endpoints. For primary producers, only endpoints with mortality or growth as the effect measurement were used as short-term data. For all other species, only endpoints from the mortality group as effect measurement were used as short-term data.

Long-term endpoints were defined for all organisms (i.e. both primary producers and others) as “$AC_{10}$”, "$EC_{10}$", "$IC_{10}$", "$AD_{10}$", "$ED_{10}$", "$ID_{10}$", "$LD_{10}$", "NOEC" or "NOEL" values based on effect measurements from the growth, development or reproduction group, and with a test duration of ≥ 2 d.

### *5.2.3 PNEC calculation and choice of assessment factors*

Predicted no-effect concentrations were calculated by dividing the lowest effect concentration per substance by an AF determined based on the data availability per substance. AFs were calculated in a sequential approach based on 7 criteria described in **Table 5.1**. These conditions were based on the European Commission’s technical guidance document (TGD) for deriving EQS [6]. While the TGD for deriving EQS makes a distinction between species from different trophic levels for the selection of assessment factors (AFs), our code refers to species belonging to different species groups. These species groups might in specific cases belong to the same trophic level, which is a difference with the TGD for deriving EQS. In our code, “additional marine species groups” are defined as species groups other than algae,

crustaceans or fish that have not yet been considered for the fulfillment of a previous criterion, restricted to those data that have been obtained by tests in a salt water environment (as indicated in the database entries).

**Table 5.1 Conditions used for the determination of assessment factors for PNEC calculations for each individual substance. The selected assessment factors were based on the Technical Guidance document for deriving Environmental Quality Standards [6].**

| Condition | Data availability criterium | Assessment factor |
|---|---|---|
| 1 | At least 3 short-term endpoints for species representing three different species groups (as defined under section 5.2.1.2). | 10,000 |
| 2 | At least 3 short-term endpoints for species representing three different species groups plus 2 short-term endpoints for additional marine species groups other than algae, crustacean and fish. | 1,000 |
| 3 | At least 1 long-term endpoint. | 1,000 |
| 4 | At least 2 long-term endpoints from species representing two different species groups. | 500 |
| 5 | At least 3 long-term endpoints from species representing two different species groups. | 100 |
| 6 | At least 3 long-term endpoints for species of three different species groups plus 1 long-term endpoint for an additional marine species | 50 |
| 7 | At least 3 long-term endpoints for species of three different species groups plus 2 long-term endpoints for additional marine species | 10 |

AFs were defined based on the number of conditions the dataset fulfilled per individual substance by sequentially checking conditions 1 to 7, e.g. if conditions 1-4 were fulfilled by the dataset for substance x and condition 5 was not, the AF = 500. In case the basic dataset (condition 1, i.e. at least 3 short-term endpoints for species representing three different species groups) was not fulfilled but a long-term endpoint was available, an AF of 1,000 was applied to the most sensitive long-term endpoint. If less than 3 short-term endpoints and no long-term endpoints were available, no AF was assigned and accordingly no PNEC was derived. Ultimately, a substance-specific PNEC was calculated by dividing its lowest available endpoint by the selected AF.

### *5.2.4 Screening-level risk assessment*

#### *5.2.4.1 Priority pollutants with existing PNEC or EQS*

For a number of priority pollutants either EQS or PNECs are available in literature [4, 72, 115]. While there exist saltwater specific AA-EQS for WFD priority pollutants (“AA-EQS for other surface waters”) [4] no specific marine PNECs exist for WFD watchlist substances [72] and most antibiotics [115]. Consequently, for the risk characterization of WFD priority pollutants we used existing AA-EQS while for the other two substance groups we first derived PNECs and

then compared with measured concentrations of the chemicals of emerging concern (CECs). RQs of each individual substance i were defined according to Equation 1.1 where PNEC was replaced by the AA-EQS where applicable.

#### *5.2.4.2 Chemicals of emerging concern without existing PNEC or EQS*

Based on the simplified automated methodology applied for PNEC calculations and therewith associated compromises we consider our risk assessment a screening-level risk assessment that could be used as a tool for prioritization of substances in further research in the BPNS.

The risk assessment combined data from our monitoring campaigns in the BPNS with the substance specific PNEC values ($PNEC_i$) calculated based on ecotoxicity data from the US EPA ECOTOX knowledgebase [80]. The monitoring data was separated according to grab water sampling and passive sampling (both expressed as water concentrations, $C_i$) and substance-specific RQs calculated using ***(Eq. 1.1)***.

Grab water sample data was summarized as the average concentration measured during sampler deployment and retrieval. For passive sampler data, speedisk extract concentrations were transformed into environmental concentrations (in marine water) by using a substance-specific partitioning coefficient previously determined in Vanryckeghem (2020) [71]. All measurements were summarized as the average of triplicates.

## 5.3 Results

### *5.3.1 Automated PNEC calculations*

PNECs for 88 substances were calculated and values ranged from 0.0015 ng $L^{-1}$ (17β-Trenbolone) to 18,000 ng $L^{-1}$ (metronidazole). Details about the quantity and type of ecotoxicity data available and used for PNEC derivation can be found in annex B (Table B1). An overview of the PNEC distribution is given in ***Figure 5.1***. Overall, the most sensitive species groups for PNEC derivation were fish, crustaceans and algae as shown in **Figure 5.2**.

### *5.3.2 PNEC calculation including ecotoxicity data from chapter 4*

As described in Chapter 4, we generated marine ecotoxicity data for a selection of 23 substances to fill data gaps in the US EPA Ecotox Knowledgebase. In an additional scenario, we included the newly generated ecotoxicity data into the automated PNEC calculation. This allowed the calculation of a PNEC for 5 additional substances (alachlor, amantadine, metoprolol, moxifloxacin and venlafaxine) for which a PNEC calculation solely using the US EPA ecotoxicity data was not possible. For another 3 substances (sodium diclofenac, flufenacet and oxytetracycline), including our ecotoxicity data lead to a refinement of the PNEC due to a reduced AF. An overview of all changes is given in ***Table 5.2***.

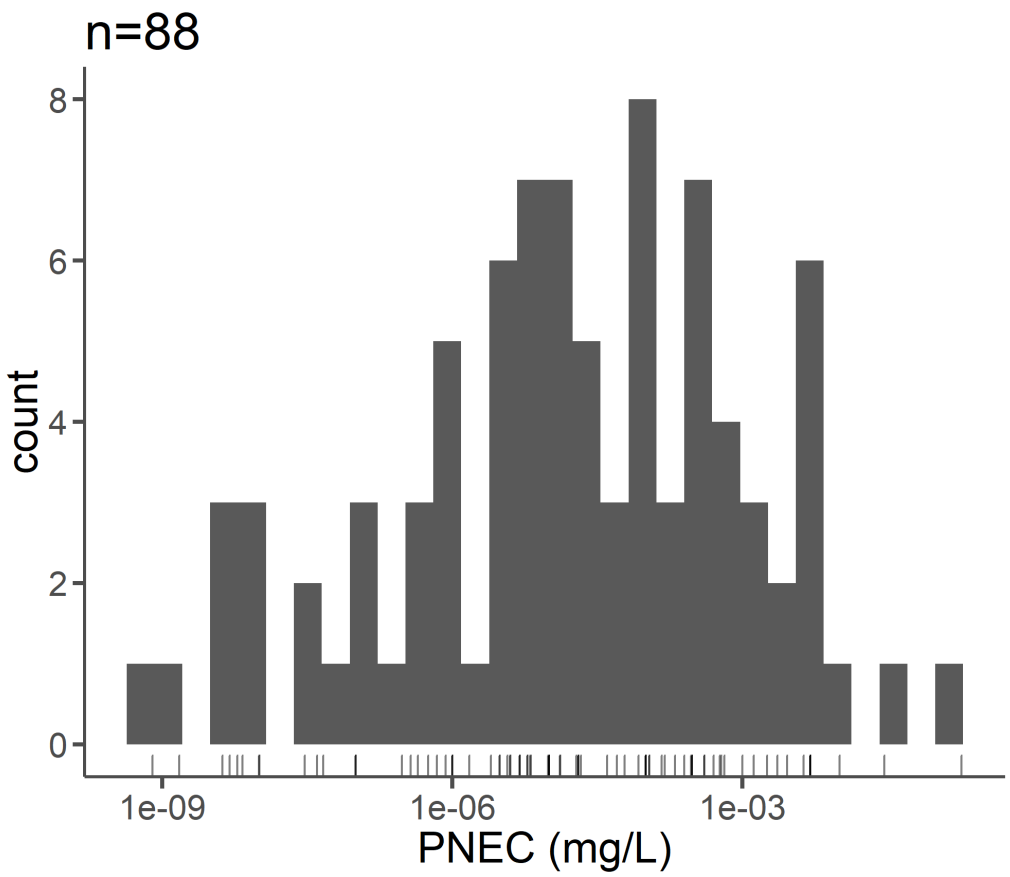


**Figure 5.1 Distribution of calculated PNECs for 88 substances with automated screening-level method based on data retrieved from the US EPA ECOTOX Knowledgebase.**

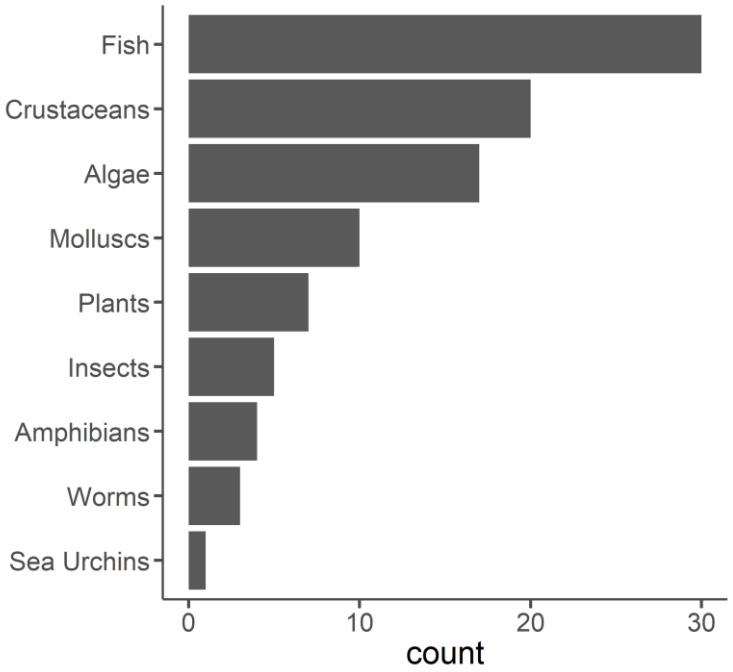


**Figure 5.2 Frequency distribution (across all substances) of the organism group with the most sensitive species.**

### *5.3.3 Risk characterization for substances with existing EQS or PNEC*

Probability distributions of RQs for 7 WFD priority substances, 7 WFD watchlist substances, and 7 antibiotics across all monitoring stations and campaigns, and for both grab samples (median of deployment and retrieval) and passive samples are presented in **Figure 5.3**. The PNECs used for all substances considered here and summaries of the RQ values are available in ***Table 5.3***, ***Table 5.4*** and ***Table 5.5***.

Among the 7 WFD priority pollutants, none showed RQ > 1 for grab samples, with median RQ > 0.1 only for diethyl-hexyl-phthalate (DEHP), cybutryne and terbutryn. For passive samples, none of the WFD priority substances showed RQ > 1, with median RQ > 0.1 only for terbutryn.

Among the 7 WFD watchlist substances, 2 showed RQ > 1 for grab samples, i.e. 17α-Ethinylestradiol (EE2) (median RQ = 36) and 17β-Estradiol (E2) (median RQ = 19), and 1 showed RQ >0.1, i.e. Estrone (E1). For passive samples, 2 WFD watchlist substances showed RQ > 1, i.e. EE2 (median RQ = 11) and E2 (median RQ = 88) and 1 showed RQ > 0.1, i.e. azithromycin.

Among the 15 antibiotics, none showed RQ > 0.1 for grab samples. For passive samples 1 antibiotic showed RQ > 0.1, i.e. azithromycin, and all others showed RQ < 0.01.

Overall, **Figure 5.3** suggests higher RQs based on grab samples than based on passive samples, with the difference being more pronounced for WFD priority and WFD watchlist substances, and with no obvious difference for antibiotics.

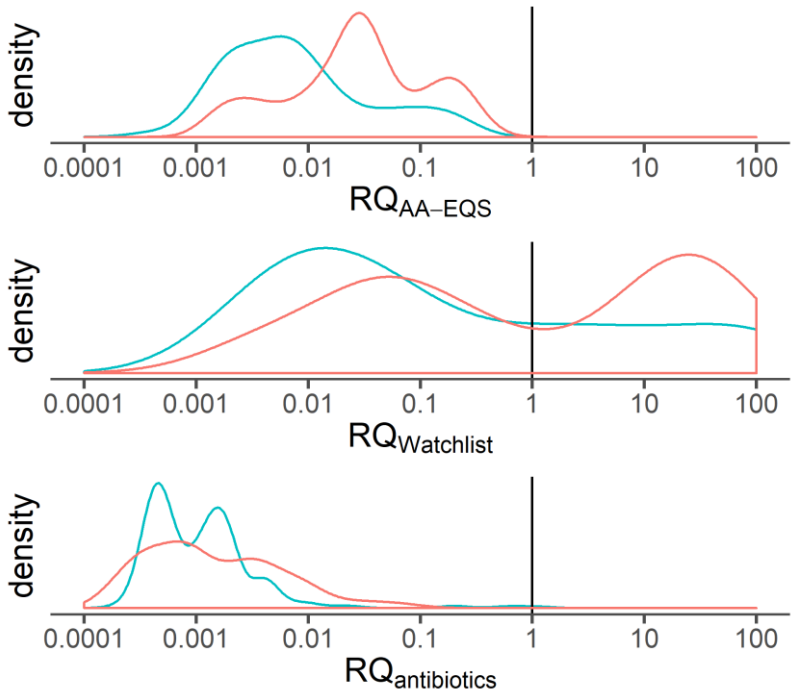


**Figure 5.3 Probability distributions of risk quotients for 7 WFD priority pollutants ($RQ_{AA-EQS}$), 7 WFD watchlist substances ($RQ_{watchlist}$), and 18 antibiotics ($RQ_{antiobiotics}$) across all monitoring stations and campaigns for grab samples (red) and speedisks (green).**

**Table 5.2 Overview of substances with changes to the PNEC due to merging of in-house generated ecotoxicity data in this thesis (Chapter 4) and data from the US EPA ECOTOXicology Knowledgebase. Changes are marked in bold and replaced values are crossed out.**

| Substance | CAS | Most sensitive species | Species group of most sensitive species | Lowest effect concentration (µg $L^{-1}$) | Effect parameter | Test duration (d) | Endpoint | Number of acute endpoints | Number of chronic endpoints | AF | PNEC (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Alachlor | 15972608 | *Phaeodactylum tricornutum* | Algae | 3,600 | $EC_{10}$ | 3 | Growth inhibition | 2 | 1 | ~~**NA**~~ **-> 1000** | ~~**NA**~~ **-> 3.6** |
| Amantadine | 768945 | *Phaeodactylum tricornutum* | Algae | 3,500 | $EC_{10}$ | 3 | Growth inhibition | 3 | 1 | ~~**NA**~~ **-> 1000** | ~~**NA**~~ **-> 3.5** |
| Sodium diclofenac | 15307796 | *Danio rerio* | Fish | 10 | NOEC | 14 | Progeny counts | 4 | 8 | ~~**1000**~~ **-> 100** | ~~**0.01**~~ **-> 0.1** |
| Flufenacet | 142459583 | *Lemna gibba* | Plants | 0.44 | NOEL | 14 | Abundance | 7 | 6 | ~~**1000**~~ **-> 100** | ~~**0.00044**~~ **-> 0.0044** |
| Metoprolol | 51384511 | *Phaeodactylum tricornutum* | Algae | 400 | $EC_{10}$ | 3 | Growth inhibition | 4 | 1 | ~~**NA**~~ **-> 1000** | ~~**NA**~~ **-> 0.4** |
| Moxifloxacin | 354812412 | *Phaeodactylum tricornutum* | Algae | 23,000 | $EC_{10}$ | 3 | Growth inhibition | 2 | 1 | ~~**NA**~~ **-> 1000** | ~~**NA**~~ **-> 23** |
| Oxytetracycline | 2058460 | *Anabaena cyclindrica* | Algae | 3.1 | NOEC | 6 | Abundance | 6 | 22 | ~~**1000**~~ **-> 50** | ~~**0.0031**~~ **-> 0.062** |
| Venlafaxine | 93413695 | *Phaeodactylum tricornutum* | Algae | 6,900 | $EC_{10}$ | 3 | Growth inhibition | 2 | 1 | ~~**NA**~~ **-> 1000** | ~~**NA**~~ **-> 6.9** |

**Table 5.3 Summary of the calculated risk quotients (RQ) per substance based on WFD priority pollutant AA-EQS for "other surface waters". N indicates the number of detects above the method quantification limit. The total number of grab samples and speedisk-derived samples were 16 and 12, respectively. RQ values > 0.1 are marked in *bold italic*.**

| Substance | CAS number | AA-EQS for "other surface waters" (ng $L^{-1}$) | Sampling method | $RQ_{median}$ | $RQ_{max}$ | N |
|---|---|---|---|---|---|---|
| Atrazine | 1912249 | 600 | grab sampling | 0.0023 | 0.0060 | 16 |
| Atrazine | 1912249 | 600 | speedisks | 0.0020 | 0.0052 | 12 |
| Cybutryne | 28159980 | 2.5 | grab sampling | ***0.12*** | ***0.40*** | 12 |
| Cybutryne | 28159980 | 2.5 | speedisks | 0.063 | ***0.23*** | 12 |
| Di(2-ethylhexyl) phthalate | 117817 | 1,300 | grab sampling | ***0.22*** | ***0.31*** | 14 |
| Di(2-ethylhexyl) phthalate | 117817 | 1,300 | speedisks | 0.0017 | 0.0029 | 9 |
| Diuron | 330541 | 200 | grab sampling | 0.019 | 0.062 | 13 |
| Diuron | 330541 | 200 | speedisks | 0.0076 | 0.018 | 13 |
| Isoproturon | 34123596 | 300 | grab sampling | 0.010 | ***0.14*** | 16 |
| Isoproturon | 34123596 | 300 | speedisks | 0.0066 | 0.048 | 12 |
| Pentachlorophenol | 87865 | 400 | grab sampling | 0.031 | 0.047 | 7 |
| Terbutryn | 886500 | 6.5 | grab sampling | ***0.18*** | ***0.22*** | 6 |
| Terbutryn | 886500 | 6.5 | speedisks | ***0.18*** | ***0.26*** | 4 |

**Table 5.4 Summary of the calculated risk quotients (RQ) per substance based on WFD watchlist PNECs. N indicates the number of detects above the method quantification limit. The total number of grab samples and speedisk-derived samples were 16 and 12, respectively. RQ values > 0.1 are marked in *bold italic* and RQ > 1 in bold.**

| Substance | CAS number | PNEC (ng $L^{-1}$) | Sampling method | $RQ_{median}$ | $RQ_{max}$ | N |
|---|---|---|---|---|---|---|
| 17α-Ethinylestradiol | 57636 | 0.035 | grab sampling | **8.6** | **71** | 10 |
| 17α-Ethinylestradiol | 57636 | 0.035 | speedisks | **11** | **11** | 1 |
| 17β-Estradiol | 50282 | 0.4 | grab sampling | **3.3** | **117** | 9 |
| 17β-Estradiol | 50282 | 0.4 | speedisks | **1.5** | **126** | 7 |
| Azithromycin | 83905015 | 19 | speedisks | ***0.19*** | **1.3** | 5 |
| Clarithromycin | 81103119 | 120 | grab sampling | 0.019 | 0.053 | 4 |
| Clarithromycin | 81103119 | 120 | speedisks | 0.0017 | 0.016 | 9 |
| Clothianidin | 210880925 | 130 | grab sampling | 0.0017 | 0.027 | 14 |
| Clothianidin | 210880925 | 130 | speedisks | 0.0034 | 0.021 | 8 |
| Estrone | 53167 | 3.6 | grab sampling | ***0.14*** | *0.56* | 9 |
| Estrone | 53167 | 3.6 | speedisks | 0.00030 | ***0.11*** | 2 |
| Thiacloprid | 111988499 | 10 | grab sampling | 0.0079 | **3.3** | 15 |
| Thiacloprid | 111988499 | 10 | speedisks | 0.0037 | ***0.12*** | 9 |
| Thiamethoxam | 153719234 | 42 | grab sampling | 0.028 | **1.3** | 12 |
| Thiamethoxam | 153719234 | 42 | speedisks | 0.0054 | 0.045 | 10 |

**Table 5.5 Summary of the calculated risk quotients (RQ) per substance based on PNECs derived for antibiotics. N indicates the number of detects above the method quantification limit. The total number of grab samples and speedisk-derived samples were 16 and 12, respectively. RQ values > 0.1 are marked in *bold italic* and RQ > 1 in bold.**

| Substance | CAS number | PNEC (ng $L^{-1}$) | Sampling method | $RQ_{median}$ | $RQ_{max}$ | N |
|---|---|---|---|---|---|---|
| Azithromycin | 83905015 | 20 | speedisks | ***0.53*** | **1.2** | 5 |
| Clarithromycin | 81103119 | 80 | grab sampling | 0.061 | 0.079 | 4 |
| Clarithromycin | 81103119 | 80 | speedisks | 0.0099 | 0.024 | 9 |
| Flumequine | 42835256 | 250 | grab sampling | 0.0085 | 0.048 | 3 |
| Flumequine | 42835256 | 250 | speedisks | 0.00069 | 0.00075 | 3 |
| Metronidazole | 443481 | 130 | grab sampling | 0.0074 | 0.034 | 8 |
| Metronidazole | 443481 | 130 | speedisks | 0.0045 | 0.0079 | 4 |
| Nalidixic acid | 389082 | 16,000 | grab sampling | 0.00039 | 0.00060 | 5 |
| Nalidixic acid | 389082 | 16,000 | speedisks | 0.000038 | 0.00021 | 6 |
| Sulfamethoxazole | 723466 | 600 | grab sampling | 0.0059 | 0.024 | 16 |
| Trimethoprim | 738705 | 500 | grab sampling | 0.00086 | 0.0092 | 15 |
| Trimethoprim | 738705 | 500 | speedisks | 0.0011 | 0.0042 | 12 |

### *5.3.4 Screening-level risk assessment*

After calculating PNECs for those substances with ecotoxicity data available in the US EPA ECOTOX knowledgebase, RQs were calculated for each measurement. Below, we undertake different comparisons to i) investigate spatio-temporal distributions of RQs among the four sampling locations in the BPNS, ii) explore potential differences between grab sample-based and passive sampler-based RQs and iii) help prioritizing CECs that require further investigation.

#### *5.3.4.1 Spatio-temporal distribution*

The spatio-temporal distribution of all calculated RQs is depicted in ***Figure 5.4***. Overall, the RQs distribute very evenly among the different sampling locations with a density peak around RQ = 1.

#### *5.3.4.1 Active vs. passive sample-based risk assessment*

***Figure 5.4*** provides probability distributions of RQs for each sampling location across SC2 – SC5, based on both grab samples (mean of deployment and retrieval) and speedisks. Even though water grab sample-based RQs seem to be a little higher for many substances, overall no obvious difference of RQ distributions between the two sampling methods were observed. RQs > 1 (and even > 10 or > 100) were found at all locations and with both sampling methods. While water grab sample-based RQs for mestanolone, dibutyl phthtalate, diisodecyl phthalate, dexamethasone and S-metlachlor were found to exceed RQ = 1, passive sampler-based RQs were found to be below this threshold. The opposite, i.e. water grab sample RQ < 1 and passive sampler-based RQ > 1 was found for diethyl toluamide (DEET) and dihexyl phthalate.

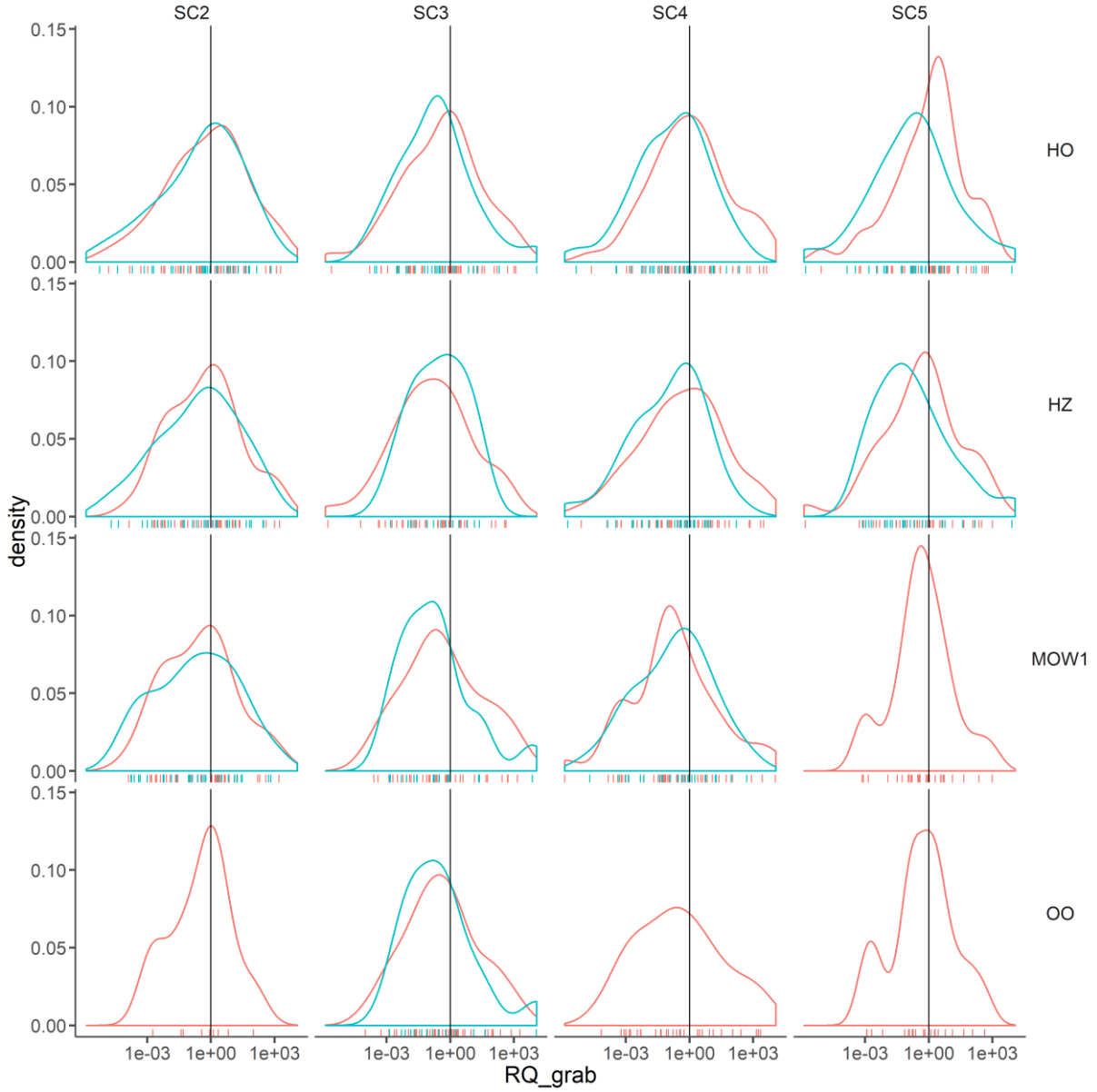


**Figure 5.4 Density plots of the calculated risk quotients (RQs) for all samples at the four sampling locations harbor Ostend (HO), harbor Zeebrugge (HZ), coastal sampling location near Zeebrugge (MOW1) and coastal sampling location near Ostend (OO) for sampling campaigns (SC) 2 – 5. Shown are the grab sample-based risk quotients in red and the speedisk-based risk quotients in green.**

#### *5.3.4.2 Prioritization of chemicals*

**Figure 5.5** provides probability distributions of RQs grouped per chemical class across all locations and SCs, and for both grab samples (mean of deployment and retrieval) and passive samples. Based on grab samples, the widest distribution of RQ appears to be for steroids, spanning > 6 orders of magnitude. All substance classes contain many cases with RQ > 1, with most RQ's for PCP's and pharmaceuticals and phthalates < 10, but with pesticides, phenols and steroids even cases > 100. The distribution of grab sample and speedisk derived RQs are comparable with each other for pesticides and pharmaceuticals, but for phenols, phthalates and steroids the grab sample-based RQs are higher than the speedisk-based RQs, while for PCPs the speedisk-based RQ's show a higher density around RQ = 1.

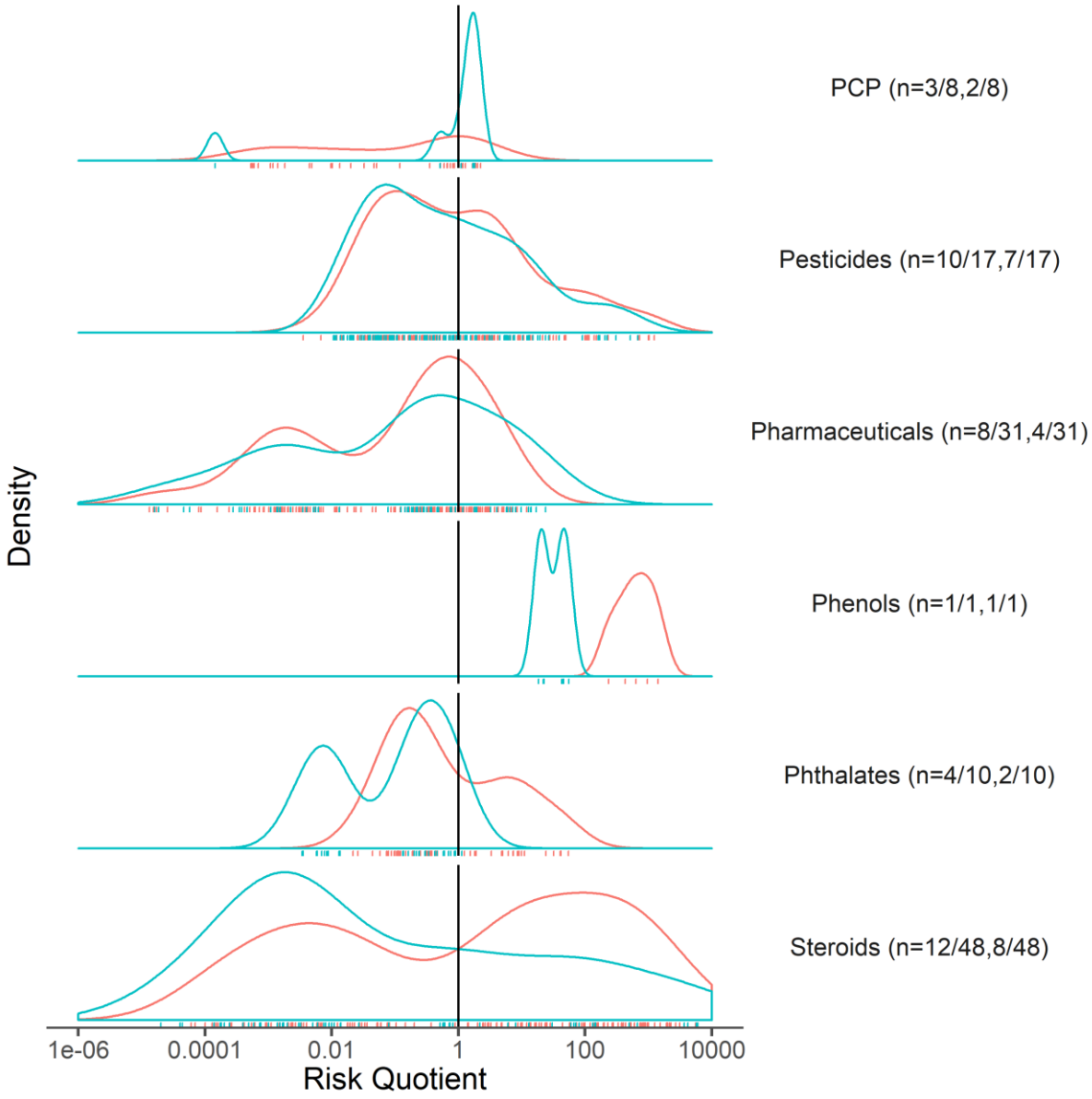


**Figure 5.5 Probability distribution of screening level risk quotients for CECs in grab samples (red) and speedisk samples (green) across sampling campaigns 2-5, per chemical class. n describes the amount of substances per compound class with an RQ > 1 for grab samples and speedisks, respectively.**

In order to shed some further light on the substances with RQ > 1, we calculated median RQ values against the PNEC (**Figure 5.6**). WFD priority pollutants were not included in this analysis, but WFD watchlist substances and antibiotics were since for none of the latter two groups PNECs specifically for marine waters exist. We found 24 substances with median RQ > 1, both water grab sample and speedisk-based. Among those, only 6 belong to the WFD watchlist substances, i.e. EE2, E2, E1, imidacloprid (IMI), clothianidin (CLO) and sodium diclofenac. Among those 24 substances, we found 10 steroids, 5 pesticides (2 neonicotinoid insecticides and 3 herbicides), 3 pharmaceuticals, 3 phthalates, 2 PCPs and Bisphenol A. Overall, this suggests that there are several substances of potential emerging concern for the marine environment that are currently not on the WFD watchlist. Finally, we also calculated RQ values at taxonomic level, with e.g. chronic $RQ_{fish}$ = median concentration in grab sample / lowest chronic NOEC or $EC_{10}$ for a fish species (**Figure 5.6**), in order to gain some additional insights

into taxonomic group sensitivity. For 4 substances we observed the MEC to exceed the chronic NOEC or $EC_{10}$ ($RQ_{taxonomic\ group} > 1$) of at least one species namely for EE2 (fish), E2 (fish), Bisphenol A (fish and mollusks) and IMI (crustaceans). Further, it is shown that most PNECs are derived from long-term data. In addition, it appears that especially for substances with higher RQ's (including for many steroids), fish are often the most sensitive taxonomic group. However, among all substances with RQ > 1, various taxonomic groups appear to be the most sensitive (**Figure 5.6**).

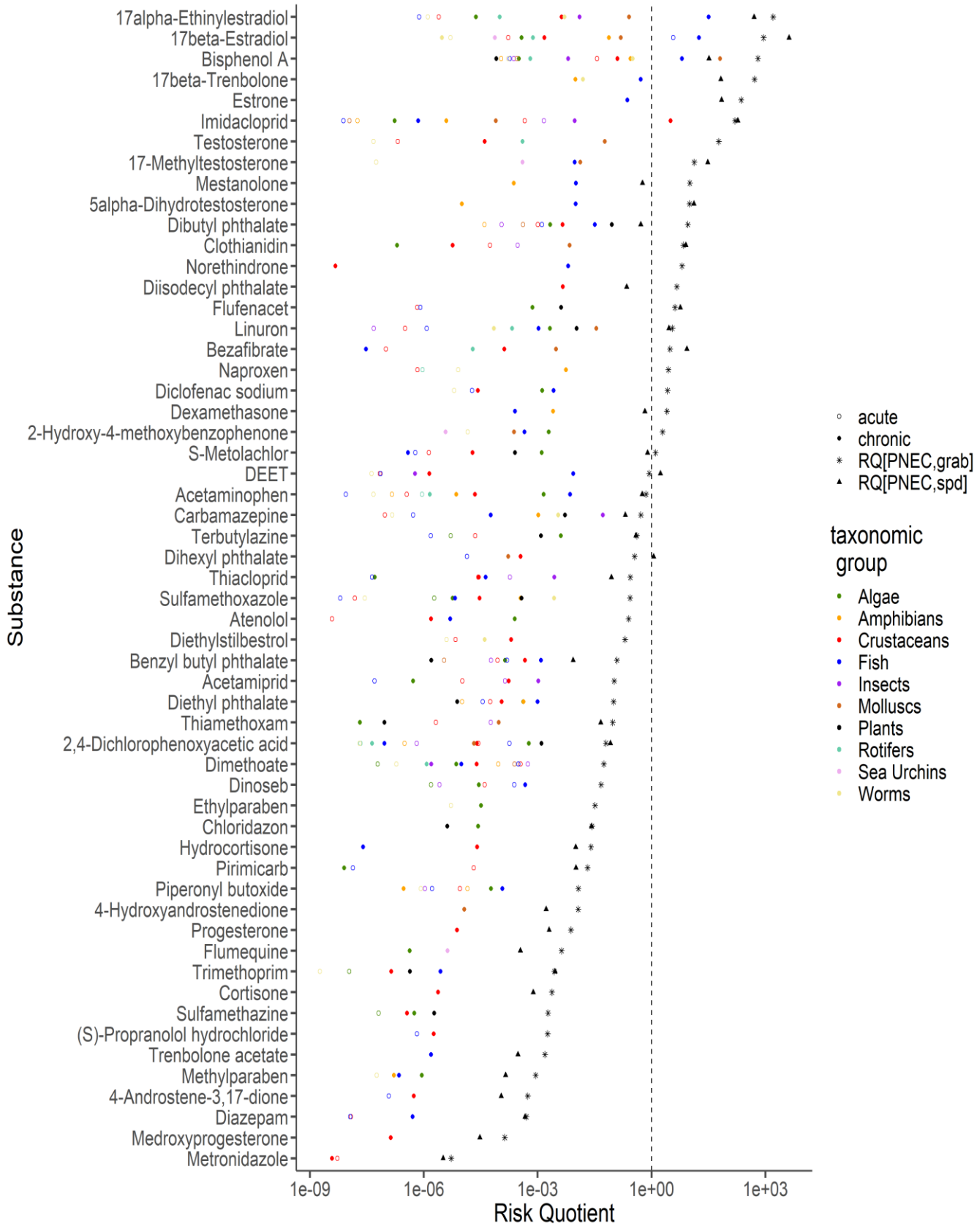


**Figure 5.6 Overview of the risk quotients (RQ) against either the screening-level PNEC as effect measurement, or against the lowest acute $LC_{50}$ or $EC_{50}$ or chronic NOEC or $EC_{10}$ per taxonomic group for which ecotoxicity data were available.**

## 5.4 Discussion

### *5.4.1 The automated calculation algorithm*

The here developed calculation algorithm for automated PNEC derivation and screening-level risk assessment should not be considered definitive. For the sake of automatization, PNECs have been estimated using a simplified (and easily automatable) set of rules compared to the PNEC derivation for marine environments under the EU's TGD for deriving EQS [6]. Rather, they should be used to provide an overall picture and to prioritize certain sampling locations, certain chemicals or certain classes of chemicals for closer inspection and detailed refinement of PNEC and RQ calculations (such as performed under Chapter 4).

At the beginning of the code, we implemented a set of filtering steps to guarantee data uniformity and quality. These filtering steps reduced the data entries from initially 32,626 to 7,402. More than 20,000 entries were not conform with our effect level requirements, i.e. other than 10 or 50 percent concentrations/doses or other than NOEC or NOEL. The TGD for deriving EQS is clear in which effect levels can be used for risk assessment purposes and removing this filtering step was out of question.

### *5.4.2 Automated PNEC calculations*

The actual PNEC calculations faced two major challenges. First, the PNEC calculation is highly dependent on the AF in use, that itself depends on the data availability. While the TGD for deriving EQs discriminates between species from different trophic levels for the AF definition, the data entries from the US EPA ECOTOXicology Knowledgebase did not allow such discrimination due to missing information. Instead, we defined 11 species classes regardless of their trophic level and representing > 98.5 % of the data entries. The second challenge was associated with the discrimination between short-term and long-term data since this is another crucial classification for the determination of the AF and thus the calculation of PNECs. The challenge here is that given the huge diversity of test organisms, biotests systems and effect measurements it is impossible to define specific criteria for all possible combinations. Instead, we discriminated between primary producers (algae and plants) and all other species classes and separated into short-term and long-term endpoints by means of the reported test duration. The limits (4d and 7d for other species classes and primary producers, respectively) were based on frequently used Organization for Economic Cooperation and Development **(**OECD) or ISO standard test guidelines [34, 116, 117].

Other than that, the PNEC calculation was relatively close to the description in the TGD for deriving EQS [6] with one exception. For the case of substances with less than 5 acute endpoints of which two for marine species (condition 2) but an available long-term endpoint, we neglected the missing acute data and derived a PNEC based on the long-term endpoint applying an AF of 1,000. This seemed reasonable since we aimed to provide a screening-level risk assessment approach for prioritization of substances for further research and in this case a PNEC derived based on few data entries is preferred over no available PNEC for a specific substance.

### 5.4.3 Including in-house data for PNEC calculations

Including in-house marine ecotoxicity data generated in Chapter 4 led to the calculation of PNECs for 5 additional substances and uncertainty reduction for another 3 substances by reducing the AF. Here, the calculation of additional PNECs was only possible due to our special case where the availability of one long-term endpoint could be used to derive a PNEC applying an AF of 1,000 to the lowest available endpoint. A look at the most sensitive species groups in **Figure 5.6** reveals that these only rarely include mollusks, sea urchin or worms which are typically represented by marine species. In general, the ecotoxicity data for marine species from Chapter 4 was successfully implemented to derive PNECs for 5 additional compounds and underlines the general lack of ecotoxicity data for marine species. The adjustment of the PNEC for another 3 substances suggests that generating ecotoxicity data for marine species for a broad set of compounds could lead to a reduced uncertainty by lowering the AF used for PNEC derivation.

### 5.4.4 Risk characterization for substances with existing PNECs

Overall, risk characterization for chemicals with existing PNECs resulted in exceedance of RQ = 1 for the two watchlist substances EE2 and E2. These two substances have reported PNECs of 0.035 ng $L^{-1}$ and 0.4 ng $L^{-1}$, respectively [72] based on very high sensitivity of fish for these hormones [118]. Both substances showed relatively high abundances across all SCs and locations and in combination with their low PNECs confirmed their status as priority pollutants.

### 5.4.5 Spatio-temporal distributions

The RQ distributions shown in ***Figure 5.4*** revealed no obvious spatio-temporal differences in the BPNS. In a monitoring study for 13 pharmaceuticals at the BPNS Wille et al. (2010) reported relatively high temporal variations for some of the target substances, e.g. maximally factor 6.4, 8.5, 12 and 16 for salicylic acid at OZ_MOW1, HZ, HO and OO_X, respectively. For other substances, the variations were comparably low as in our study with a factor ≤ 4 across different sampling locations for e.g. carbamazepine, bezafibrate or propranolol [119]. From a purely chemical analytical perspective Vanryckeghem (2020) reported on average 4 and 3 times higher concentrations of the NewSTHEPS target compounds at HO as compared to HZ based on grab water samples and passive samples, respectively [71]. For the comparison of the respective harbor and coastal locations a factor 2 and 10 higher concentrations in the harbors for Zeebrugge and Ostend were reported, respectively [71]. Thus, while especially local variations of the chemical's concentrations were observed, these are not directly observed from a risk assessment-based perspective. Indeed, while Vanryckeghem (2020) directly compared chemical concentrations we look at density distributions for RQs across all substances and detects.

### 5.4.6 Active vs. passive sampler-based risk assessment

A comparison of the RQs defined for the two sampling methods resulted in slightly higher grab sample-based RQs for most substances with the majority of RQs being within a factor 10 as shown in **Figure 5.6**. This is in agreement with the water grab sample concentrations being on average a factor 3 higher than passive sampler-based concentrations [71], a ratio that has

been confirmed for similar passive sampler configurations and substances [120, 121]. This might be linked to the fact that grab sampling usually results in the measurement of the total chemical concentration in a water body while passive sampling rather samples the bioavailable fraction [122].

### *5.4.7 Screening level risk assessment for the BPNS*

Screening-level risk assessment identified 24 substances with median RQ > 1, of which only 6 are WFD priority pollutants and thus included in regular monitoring efforts. Among these 6 substances, we found 2 neonicotinoid insecticides, i.e. CLO and IMI. In the case of IMI this clearly confirms our findings illustrated in Chapter 4 of this thesis where IMI was found to be the neonicotinoid insecticide with most exceedances of RQ = 1. For CLO on the other our results from Chapter 4 suggested it to be the least harmful substances among the four tested neonicotinoids. The higher risk found in the automated screening-level risk assessment is related to the comparably low derived PNEC of 0.1 ng $L^{-1}$ (automated PNEC derivation) vs. 250 ng $L^{-1}$ (AA-EQS derivation in chapter 4). The automated PNEC for CLO was derived with an AF of 1,000 based on chronic mollusk data while the EQS derivation was based on our own (sub)chronic copepod data. The example of CLO also shows the advantage of an in-depth threshold derivation allowing the consideration of various sources of ecotoxicity data. On the other hand, this is linked to a higher time consumption and as such not suited for a broad range of substances.

Next to neonicotinoid insecticides, three herbicides, i.e. flufenacet, linuron and s-metolachlor were found to exceed RQ = 1. For flufenacet and S-metolachlor the most sensitive species groups were plants and algae, respectively. This is logic since herbicides are designed to act on primary producers. Surprisingly, for linuron the most sensitive species group was mollusks. Typically, no direct effects of herbicides on mollusks at realistic environmental concentrations are expected but they might be indirectly affected by biomass reduction of their feed (algae and/or plants) [123]. Likewise to our findings, in a study for the development of a river basin management plan with regards to prioritization of pesticides to include as target substances, Tsaboula et al. (2016) identified linuron, S-metolachlor and IMI as potential candidates [124]. This prioritization was based on several environmental and ecotoxicological aspects, i.e. frequency of exceedances at concentrations above the PNEC, a weighting factor for the maximal exceedance of the PNEC, a criterium for the spatial distribution per substance and a Persistent, Bio-accumulative, Toxic (PBT) assessment [124].

In addition to the pesticides, potential risks were detected for 10 steroids including commonly known problematic substances such as e.g. EE2, E2 and E1 but also less studied compounds such as e.g. 17β-Trenbolone or Norethindrone. From an ecotoxicological perspective, steroids are strongly associated with endocrine disrupting effects. As such many steroids have shown to interact with the endocrine system of fish at very low concentrations (often below 1 ng $L^{-1}$) and cause deleterious effects on fish populations [118, 125, 126]. In a risk-based prioritization of pharmaceuticals for the aquatic environment, E2 and diclofenac were scored with a risk > 1 and > 10, respectively for chronic risks in the aquatic environment [127].

Overall, the screening-level risk assessment presented here, can be used to prioritize certain substances and further testing with sensitive taxonomic groups on a substance-by-substance or on a chemical class basis (e.g. steroids for fish).

## 5.5 Conclusion

Based on RQ size, this screening-level marine risk assessment suggests to prioritize in future work Bisphenol A, certain pesticides and steroids for further ecotoxicological testing and/or refined PNEC calculation, but specific substances in the PCP, phthalate or pharmaceuticals class should also not be neglected (see annex B, Tables B2 and B3 with RQ overview per substance). Further research should initially occur on a substance-per-substance basis, since with so many RQ's > 1 for individual substances performing mixtures assessment with measured targeted substances is not necessarily meaningful. The information presented here, can be used to prioritize certain substances and further testing with sensitive taxonomic groups on a substance-by-substance or on a chemical class basis (e.g. steroids for fish).

In a policy brief, Posthuma et al (2019) made recommendations on how to improve single substance-based risk assessment as currently suggested by legislation [128]. Amongst others, they suggested to move away from focusing risk assessment on a limited number of priority pollutants with existing EQS [128]. Our code not only offers a tool for automated screening-level risk assessment but also includes a module for an automated PNEC derivation based on existing ecotoxicity data. Indeed, this is currently limited to data from the US EPA ECOTOXicology Knowledgebase but offers potential for the implementation of data from other ecotoxicity databases. Of course, while the automated approach offers the possibility to move away from a strictly priority pollutant-focused risk assessment, it is still limited by the number of substances targeted in the environmental monitoring. Again, there is potential to extend the list of target substances but ultimately there will always be substances that are not included because they are either not targeted, not detected or simply unknown. Yet, these disregarded substances may contribute to eventual mixture effects and to a potential risk for the environment. Thus, a single substance-based risk assessment will always be biased towards a specific number of target substances (priority pollutants or not) and can never fully investigate the likelihood of impacts from pollution with complex chemical mixtures [63, 128].

The use of an automated calculation algorithm for PNEC derivation and screening-level risk assessment based on literature ecotoxicity data and monitoring data may facilitate chemical prioritization for further investigation. The classic derivation of PNECs or EQS values (as performed in chapter 4) is a time-intensive exercise that requires thorough argumentation and expert judgement. Automating this process is a step forward in dealing with the multitude of chemicals that has been accumulating in the marine environment. Nevertheless, it remains a target substance driven approach that is unable to account for chemicals that are unknown or present below their respective detection limit [128]. In addition, it bears the risk of neglecting interactive effects of chemicals. Indeed, existing chemical legislations are based on a substance-by-substance risk assessment. Yet, environmental risk assessment is meant to assess the real impact on ecosystems or species that are exposed to chemicals and for most of our waters this means simultaneous exposure to various chemicals. This indicates that there is a need for mixture-based risk assessment methods. To answer this need, we developed a novel method for passive sampler-based ecotoxicity testing of ERCMs in chapter 6.

# 6

# Development of a novel method for passive sampler-based ecotoxicity testing of environmentally realistic chemical mixtures

Redrafted from:

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# 6 Development of a novel method for passive sampler-based ecotoxicity testing of environmentally realistic chemical mixtures

## 6.1 Introduction

The simultaneous presence of a high number of chemicals in the Belgian Part of the North Sea (BPNS) [71] and therewith associated risks (as shown in Chapter 5) raise the question whether current environmental risk assessment practices (usually focused on single substance effects) [37] can account for the simultaneous occurrence of multiple chemicals. Contrarily to the simple procedure of ecotoxicity testing with individual substances, biotesting of environmentally realistic chemical mixtures (ERCMs) is complex and requires considerable adaptations of standardized biotest procedures. Transferring ERCMs from the field to the lab for both chemical analysis and biotesting remains a challenge, but advances in passive sampling have opened new possibilities in the recent years [129]. Passive sampling allows transferring ERCMs into biotest systems by either passive dosing (for equilibrium based samplers) or extract spiking (for integrative samplers) [129]. Applying passive sampler extract spiking in bioassays such as the 72 h growth inhibition test with *P. tricornutum* provides the opportunity of testing mixtures at a relatively high level of biological organization. Another advantage of working with passive sampler extracts rather than passive dosing is the possibility to test ERCMs at a range of relative enrichment factors (REFs) [36]. To this moment, only a limited number of ecotoxicological studies have applied one of the two methods for transferring ERCMs from the field to the lab in algae growth inhibition testing [130, 131]. Interestingly exposure of marine diatoms to realistic mixture concentration levels ranged from 50 % growth stimulation to 100 % growth inhibition and the authors concluded that “exposure to low levels of persistent organic pollutants may threaten sensitive genotypes and benefit healthy populations” [131]. This argument could be extended to stimulation effects stating that stimulation effects favoring the growth of one species may limit the growth of competing species.

When it comes to identification of the main drivers of observed ecotoxicological responses to ERCMs, sound and highly sensitive analytical methods are needed. The broad chemical diversity present in the marine environment covers many compound classes and even though a high number of compounds has already been identified, an even higher number remains unknown. In a recent review with recommendations for a more efficient assessment of chemical contamination, virtual effect-directed analysis (EDA) has been suggested as a method to reduce the complexity of chemical mixtures. Multivariate statistics could be used instead of sample fractionation to identify chemical signals correlating with observed effects [132].

In this study we investigated whether or not the growth of Phaeodactylum tricornutum is affected when exposed to ERMCs originating from passive sampler extracts. Because we observed different biological responses when repeatedly testing these extracts (see results), we then tried to determine the mixture effect drivers by applying multivariate statistics to identify differences in the mixture composition of various extracts based on 88 target compounds, comprising personal care products (PCPs), pesticides and pharmaceuticals.

## 6.2 Materials and methods

### *6.2.1 Passive sampling and sampler treatment*

For this investigation, we used speedisk passive sampler and active grab sampling data from sampling campaign 1 (SC1). Speedisks were rinsed and extracted as described in Chapter 3. Next, the extraction solvent was fully evaporated under a gentle nitrogen stream at 25 °C. Finally, the precipitate was reconstituted in 1 mL of HPLC methanol:HPLC water (10:90, v/v) acidified with 0.1 % formic acid and 0.01 % $Na_2EDTA.2H_2O$. The reconstituted extract was ultra-sonicated for 1 min, vortexed for 20 s and centrifuged at 3,000 rpm for 5 min. The liquid phase was transferred to a HPLC vial and stored at – 20 °C until instrumental analysis or biotesting. Prior to biotesting, concentration series of the passive sampler extracts were prepared by diluting the extracts in 5-fold steps. For this purpose, 90 µL original extract was diluted by adding 360 µL of the reconstitution solvent. This was repeated 8 more times to obtain a total number of 10 concentration treatments (CTs) per Speedisk extract. The exposure concentrations in this chapter are expressed as sum-analyte concentrations. A summary of the different steps from sampler recovery until final chemical analysis is shown in **Figure 6.1**:

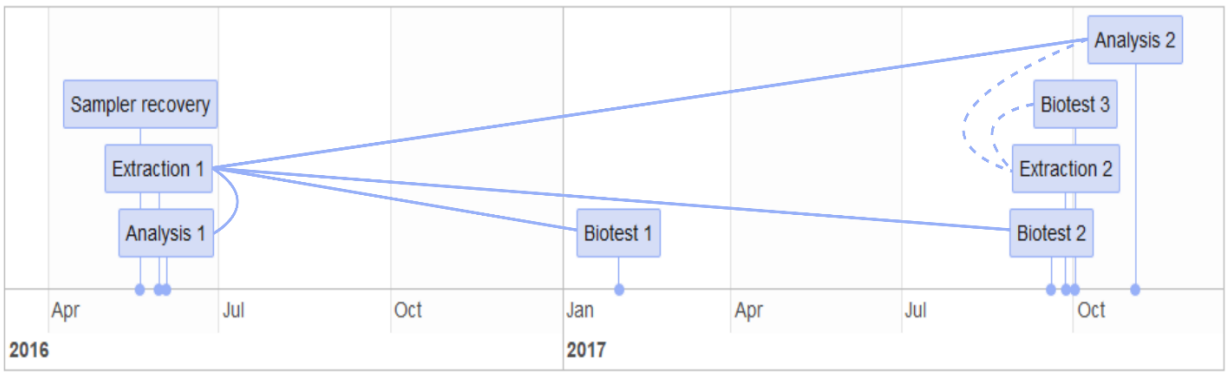


**Figure 6.1 Timeline from sampler recovery until final chemical analysis. Initial Speedisk extracts (Extraction 1) were used for biotests 1 and 2 and analyzed twice for target substances (Analysis 1 & 2) as indicated by the solid lines. Speedisk extracts of the second batch (Extraction 2) were used for biotest 3 and analyzed once (Analysis 2) as shown by the dashed lines.**

### *6.2.2 Algae growth inhibition testing*

In the initial experiments with ERCMs, we spiked 50 mL algae growth medium in erlenmeyer flasks (Biotest system 2) with 100 µL speedisk extract following the previously prepared concentration series. In addition to control flasks (see Chapter 2.2) 6 or 7 flasks were filled with growth medium and spiked with 100 µL of the reconstitution solvent to serve as solvent controls. For biotests 1 and 2, we also included procedural blanks, speedisks treated equally to the ones deployed in the BPNS but kept 67 days in deionized water. For biotest 3 there was not sufficient volume left of the extracts of these procedural blanks to be tested along with the other Speedisk extracts.

### *6.2.3 Chemical analyses*

Following biotests 2 and 3, the triplicates of each CT were pooled and the test medium was filtered using 2.7 µm glass microfiber filters (Whatman™ GF/D, GE Healthcare). The test medium was stored in polyethylene bottles at 4 °C until analysis. For analysis of the test medium, triplicates of the 5 highest CTs were pooled and analyzed two weeks after test end. This was done to explore ERCM concentrations in biotests 2 and 3 while no analysis was performed for biotest 1. Next, we analyzed all fresh Speedisk extracts (extraction 2, used in biotest 3) four weeks after extraction (extract storing for <1 month at – 20°C) together with the initial extracts (extraction 1, stored for 16 months at -20 °C).

### *6.2.4 Statistics and multivariate analysis*

All biotest results were analyzed by first comparing the control growth rates with the solvent control growth rates applying two-tailed t-tests ($\alpha = 0.05$, with a 95 % CI) to exclude the cell growth being affected by the extract reconstitution solvent. For identification of differences in growth rates among Speedisk extract exposed algae and solvent controls, one-way ANOVA ($\alpha = 0.05$) was applied followed by Dunnett's multiple comparisons test.

Correlation coefficients for the target compound concentrations in different extracts from each sampling site were calculated in order to assure that the mixture composition did not change during Speedisk storage. In addition, we applied correlation analysis to compare the actually measured with calculated target compound concentrations in the test medium by applying Pearson correlation analysis ($\alpha = 0.05$). The respective concentrations were plotted and a least-squares linear regression with 95 % CIs was fitted to these. All statistical analyses of the biotest results and the chemical analysis were conducted using GraphPad Prism version 5.01 for Windows [133].

Multivariate data analysis was performed with SIMCA 14.1 (Umetrics, Malmo, Sweden) and applied for the measured contaminant concentrations of our 89 target compounds in the Speedisk extracts used for spiking of biotests 2 and 3. While unsupervised clustering using principal component analysis (PCA) was applied to the data of both sampling locations (harbor and sea) to find groups inherent to the data, supervised clustering using orthogonal partial least squares project to latent structures-discriminant analysis (OPLS-DA) required division of the data into two parts according to the two sampling locations. The dataset was separated according to sampling location of the Speedisk extracts in order to identify the substance(s) explaining the highest variation in contaminant concentrations between the different extracts of the same sampling location. For the PCA and OPLS-DA model development, the data set first was mean-centered by calculating the average (contaminant concentration) peak spectrum of the data set and subtracting that average from each spectrum and then pareto-scaled by weighing each variable by the square root of its standard deviation, hereby amplifying the contribution of lower concentration compounds [134]. Supervised clustering was applied to find a "classifier" for the known classes. In this case the classifier was defined as a pattern in mixture composition of the different Speedisk extracts i.e. one or several substances that had clearly different concentrations in the 0 and 16 month old extracts. The identification of target compounds explaining the main differences in mixture composition between the different Speedisk extracts was achieved by means of a multi-criteria assessment (MCA) building on a combination of variable importance in the projection (VIP) statistics and S-plot

[134]. While the VIP plots clearly rank the compounds based on their contribution to the observed variance between compared Speedisk extracts, the S-plots were found to be less relevant due to the relatively small variable size of 88 compounds.

### *6.2.5 Mixture effect driver testing*

After identification of atenolol as the substance that best explained the differences in mixture composition of the different Speedisk extracts (see *Results* section), atenolol was tested individually in a 72h algae growth inhibition test as described in the respective guideline [67]. Atenolol (CAS number 29122-68-7, purity > 98 %) was purchased from Sigma Aldrich, Belgium. Atenolol was directly dissolved in artificial seawater medium prepared according to ISO 10253 [67]. In total 18 concentration treatments were prepared following a 1:1 dilution and ranging from 100 ng $L^{-1}$ to 0.00076 ng $L^{-1}$. Each treatment was tested in triplicate. Additionally one flask per CT was prepared but not inoculated with algae to serve as blank. Six plus one (blank) control flasks were filled with non-spiked artificial seawater medium. All flasks except the blanks were inoculated with 10,000 cells $mL^{-1}$ and cell density was measured daily for 72h. Culture and test conditions were as described for the Speedisk extract testing as described under *section 2.3*.

## 6.3 Results

### *6.3.1 Ecotoxicological assessment of ERCMs*

Statistical analyses showed no significant differences ($p < 0.05$) of the growth rates between the controls and the solvent controls. For all further analyses we therefore only used the solvent controls to calculate growth stimulation/inhibition values and to perform further statistical analyses since their medium composition was identical to extract spiked treatments except for the addition of Speedisk extract.

The pH varied maximally 0.9 (7.5 – 8.4, biotest 1), 0.5 (7.7 – 8.2, biotest 2) and 0.6 (7.8 – 8.4, biotest 3) units during the tests. The temperature varied by maximum 1.0 °C (21.0 °C – 22.0 °C, biotest 1), 2.0 °C (21.1 °C – 23.1 °C, biotest 2) and 2.0 °C (20.8 °C – 22.8 °C, biotest 3) throughout the test period. Both pH and temperature variation thus fulfilled the recommended validity criteria for 72 h growth inhibition testing with *P. tricornutum* [67]. The results of all algal growth inhibition tests with Speedisk extracts are shown in **Figure 6.2**.

#### *6.3.1.1 Biotest 1 (8 months extract storage time)*

The growth of *P. tricornutum* was stimulated when exposed to Speedisk extracts (extraction 1) from both sampling locations, tested 8 months after extraction. The stimulation reached a maximum of 10.6 ± 1.9 % for the harbor and 13.7 ± 2.6 % for sea samples in the highest test concentration as shown in Figure 2. The growth stimulation was observed to be statistically significant (Dunnet's test, $\alpha = 0.05$) for the 6 and 5 highest CTs for the harbor and sea samples, respectively. The blank Speedisk extracts however showed no effects on the growth of *P. tricornutum*.

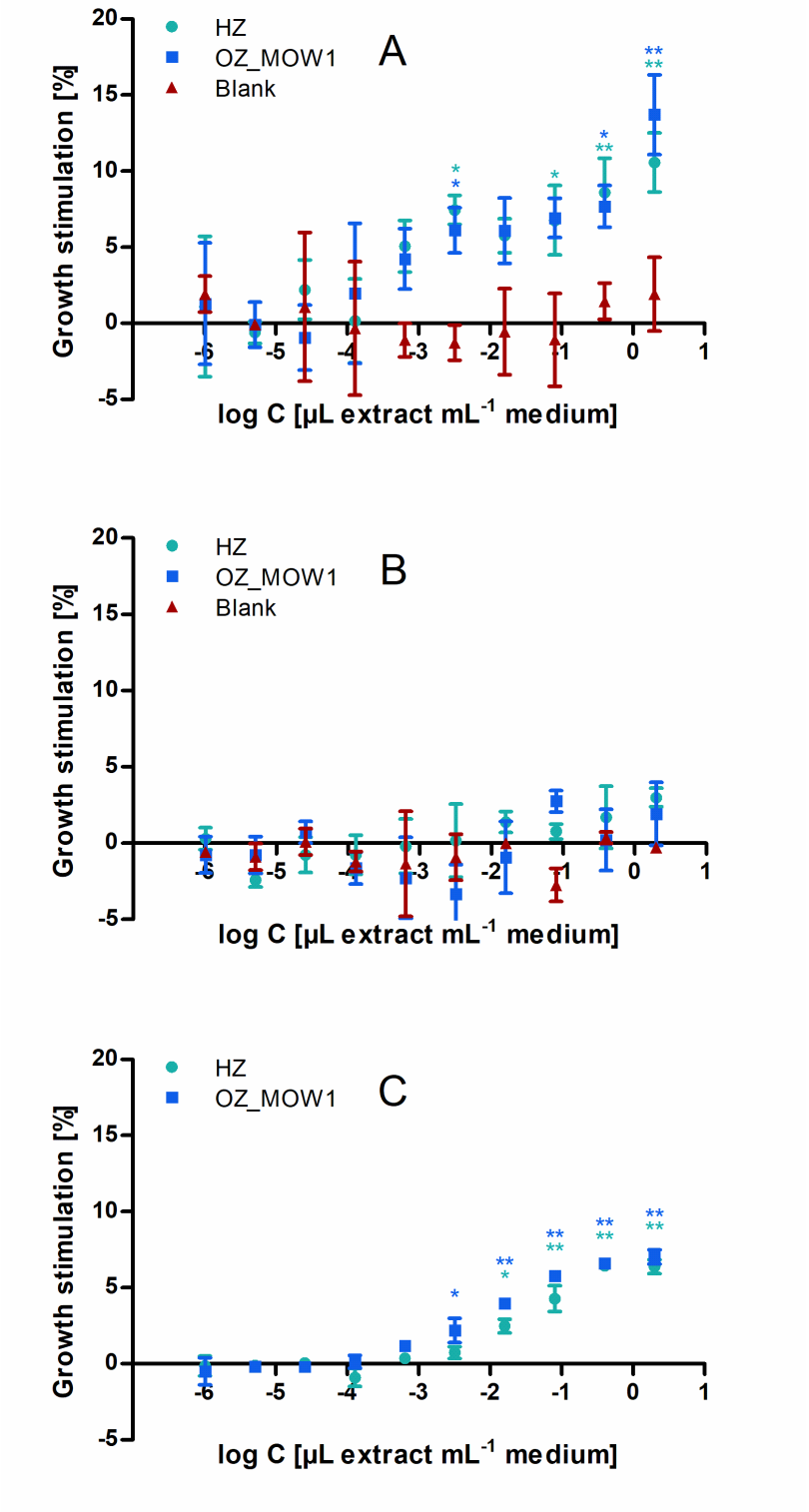


**Figure 6.2 Results of the algae growth inhibition testing at three different time points: 8 (A, biotest 1), 16 (B, biotest 2) and <1 (C, biotest 3) months after Speedisk extraction. Shown is the percentage growth stimulation versus the log concentration in µL extract per mL test medium. Data points show the mean of triplicates (biotests 1 and 2) or duplicates (biotest 3) and the standard error of the mean. An * marks significant differences in growth rate in comparison with the control treatments. HZ = Harbor Zeebrugge, OZ_MOW1 = coastal sampling location near Zeebrugge.**

#### 6.3.1.2 Biotest 2 (16 months extract storage time)

When tested after 16 months of storage time and repeated sample handling (freezing, thawing, some uncontrolled exposure to light and room temperature before biotest spiking), the extracts (extraction 1) did not exert any significant effects (Dunnet's test, $\alpha = 0.05$) on the growth of *P. tricornutum* for both sampling locations (Figure 2). Any previously observed effect disappeared completely and the growth of exposed *P. tricornutum* cells was equal to control cell growth. The same was observed for the algae cells exposed to the blank Speedisk extracts.

#### 6.3.1.3 Biotest 3 (<1 months extract storage time)

Fresh Speedisk extracts (extraction 2) with limited handling and storage time (< 1 month) between extraction and biotesting showed stimulatory effects on *P. tricornutum* for both sampling locations. The maximum stimulation was observed in the highest tested extract concentrations with 6.4 ± 0.5 % and 7.0 ± 0.5 % for harbor and sea samples, respectively. Growth stimulation was observed to be significant (Dunnet's test, $\alpha = 0.05$) for 1.3 – 159 ng $L^{-1}$ (highest 4 CTs) and 0.27 – 166 ng $L^{-1}$ (highest 5 CTs) summed target compound concentration for the harbor and sea samples, respectively.

### 6.3.2 Chemical analysis

#### 6.3.2.1 Storage of Speedisks

In order to check if storage of ERCMs bound to the Speedisk sorbent for up to 16 months did not affect the mixture composition we compared the contaminant concentration levels in the extracts of Speedisks extracted within one month after sampler recovery with the extracts of Speedisks extracted 16 months after sampler recovery. Annex C (Figure C1) shows the correlation plots for contaminants detected in both extracts. The correlation analysis for the target compound concentrations in the extracts showed significant positive correlation for both sampling locations (harbor: $R^2 = 0.67$, n = 29, $p < 0.0001$; sea: $R^2 = 0.77$, n = 30, $p < 0.0001$). A detailed list of the measured contaminant concentrations in Speedisk extracts is available in annex C (Table C1).

#### 6.3.2.2 Test medium concentrations

The test medium of the highest CT at the end of biotest 2 and biotest 3 was analyzed for the target compounds and compared to expected concentrations calculated based on the determined Speedisk extract concentrations used for spiking. Both measurements were performed simultaneously and we plotted the calculated and the measured water concentrations ($C_w$) in correlation plots (Annex C, Figure C2) and performed correlation analysis for the test medium of both sampling locations in biotests 2 and 3. All correlations were found to be significantly positive with a good fit for the $C_w$ of biotest 2 at the harbor ($R^2 = 0.79$, number of components = 10, $p = 0.0006$), a weaker fit at the sea ($R^2 = 0.46$, number of components = 19, $p = 0.0013$) and good fits for $C_w$ of biotest 3 for the harbor samples ($R^2 = 0.76$, number of components = 7, $p = 0.011$) and the sea samples ($R^2 = 0.82$, n = 16, $p < 0.0001$). In addition to this, we compared the measured and predicted test medium concentration (highest CT) with water grab samples taken during the passive sampler deployment as shown in **Figure 6.3**. This figure shows that summed contaminant

concentrations in the biotest medium were in the range of those measured in water grab samples and concentration levels in test 2 were approximately 2.5 and 1.4 times lower as compared to those in test 3 for harbor and sea samples, respectively..

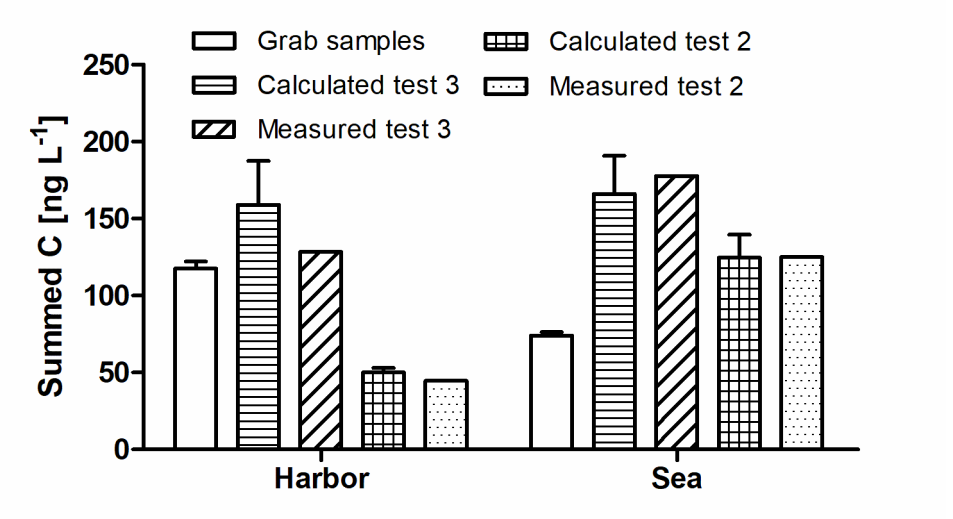


**Figure 6.3 Comparison of summed target compound concentrations (ΣC) for grab water samples and measured and calculated concentrations in biotests 2 and 3 (at the highest concentration treatment) for both sampling locations in ng $L^{-1}$. Error bars represent the standard error (SE) of the mean of triplicate measurements.**

### *6.3.3 Multivariate analysis*

Unsupervised clustering of the target substance concentrations in the extracts used in biotests 2 and 3 was applied to find groups that are inherent to the data. The applied PCA showed that Speedisk extracts grouped amongst Speedisk replicates and based on extract storage time (<1 or 16 months) for both sampling locations as shown in **Figure 6.4**. PC 1 and 2 could explain the observed variation in mixture composition of the different Speedisk extracts by 89 %. Interestingly, the mixture composition of both harbor and sea samples of biotest 3 was correlated with high concentrations (above 10 µg $L^{-1}$) of the β-blocker atenolol which presents a high impact (above 0.5) on both PCs (**Figure 6.4**). When applying supervised clustering via OPLS-DA for the harbor and sea extracts, we attained highly reliable predictive accuracy for both models as shown in ***Table 6.1*** by the values for $R^2X_{cum}$, $R^2Y_{cum}$ and $R^2Q_{cum}$, all being close to 1.0. In addition, the ANOVA for the cross-validates residuals (CV-ANOVA) performed for each model shows significant (α = 0.05) model reliability with p-values of 0.0023 and 0.019 for the harbor and sea models, respectively [135]. Next, permutation tests (100 times Monte Carlo simulation) resulted in good performance for both models with $Q^2$ intersect with the Y-axis being negative (Annex C, Figure C3). Ultimately, to identify which compound(s) was/were accountable for the variation in mixture composition an MCA was applied where first VIP statistics were used to pre-select compounds. According to the criterion for VIP statistics (VIP > 1.0), a total number of 11 and 10 compounds were obtained for their contribution to the difference in mixture composition of the Speedisk extracts. Subsequently, the S-plot (Annex C, Figure C4) and compound loading plots (Annex C, Figure C5) for the OPLS-DA models lead to the identification of atenolol as the compound contributing most to the differences in mixture composition between the Speedisk extracts causing growth stimulation versus no effect in the 72h growth inhibition experiments for both harbor and sea samples. These observations are

further supported by the measured contaminant concentrations in the extracts used in biotests 2 and 3 as shown in annex C (Table C1) with clearly decreasing concentrations in Speedisk extracts stored for 16 months for many compounds and especially atenolol.

**Table 6.1 Model parameters of the orthogonal partial least square discriminant analysis (OPLS-DA) models performed for the harbor and sea samples, respectively. $R^2X_{cum}$ and $R^2Y_{cum}$ are the cumulative modeled variation in X and Y matrix and $Q^2Y_{cum}$ is the cumulative predicted variation in Y matrix. The values of these parameters close to 1.0 indicate a robust mathematical model with reliable predictive accuracy. CV ANOVA describes the p-values of the analysis of variance of the cross-validated residuals, while the permutation tests (100 times Monte Carlo simulation) indicate the goodness-of-fit for repeated model plotting.**

| Model | $R^2X_{cum}$ | $R^2Y_{cum}$ | $Q^2Y_{cum}$ | CV ANOVA (p-value) | Permutation test |
|---|---|---|---|---|---|
| Harbor | 0.886 | 0.986 | 0.967 | 0.0023* | Good |
| Sea | 0.846 | 0.958 | 0.963 | 0.019* | Good |

* $p < 0.05$

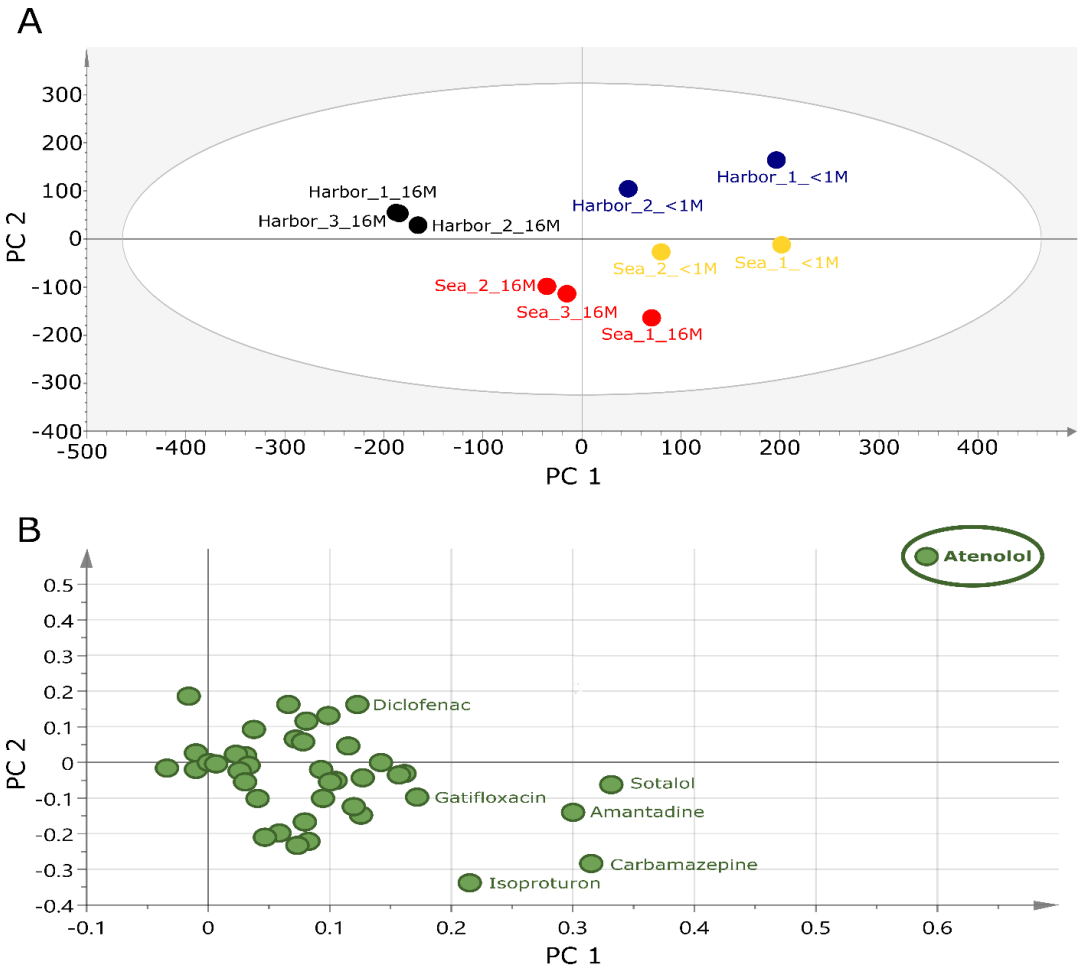


**Figure 6.4 Unsupervised clustering by principal component analysis (PCA) of all Speedisk extracts used in biotests 2 and 3. Shown are the score plot (A) and the PCA loading plot (B) of the first and second principal component (PC 1 and PC 2). 1-3 describe the respective replicate Speedisk extract and <1M and 16M indicate the time between Speedisk extraction and biotesting. PC 1 and PC 2 explain 62.2 % and 27.0 % of the observed variation, respectively. The ellipse in the PCA score plot (A) gives the 95 % confidence interval of Hotelling's $T^2$ distribution.**

### *6.3.4 Mixture effect driver testing*

Atenolol did not show significant effects (Dunnet's test, $\alpha = 0.05$) on the growth rate of *P. tricornutum* at any test concentration in comparison with the control treatments. Further details about the single substance test with atenolol can be found in annex C (Table C2). The pH and temperature varied maximally 0.7 (7.8 – 8.5) units and 2.0 °C (20.1 °C – 22.1 °C) throughout the test period. Both pH and temperature variation were thus in agreement with validity criteria for 72 h growth inhibition testing with *P. tricornutum* [67].

## 6.4 Discussion

When performing monitoring in the marine environment, researchers are depending on environmental conditions more than in any other environment. The deployment of passive sampling devices in the marine environment is linked to high costs since sampling locations are usually only reachable by ship and deployment requires specialized equipment on board. Thus, such campaigns are mostly performed to deploy a high amount of samplers to generate a maximum of data within few interventions. This leads to the fact that not all passive samplers might be processed immediately after recovery and sometimes need to be stored for a long time. This is sensitive since passive sampling aims to allow working with ERMCs, where mixture composition and contaminant concentrations are very important factors for further investigations [136]. Since we used different Speedisk extracts when spiking biotests 1+2 and biotest 3, we had to assure that both mixture composition and contaminant concentrations remained unchanged during Speedisk storage up to 16 months. Correlation analysis revealed that contaminant concentrations on the Speedisks with <1 months and 16 months of storage time after sampler recovery were significantly positively correlated ($p < 0.0001$) for both sampling locations (Annex C, Figure C1) indicating that mixture composition in replicate Speedisks remained unchanged during 16 months of storage time. The analysis was based on 30 quantified compounds each, showing a high conformity in mixture composition. These results are in good agreement with a study confirming the stability of 16 pesticides and pharmaceuticals (among them 12 substances included in our analytical method) spiked on POCIS passive samplers up to 6 years [137]. The sorbent embedded in the POCIS passive samplers is Oasis HLB, a comparable sorbent to the one in Speedisk passive samplers.

The quantification of our target compounds in the test medium after test end faced two major challenges. First, the volume of test medium used for each replicate at the test end was usually maximum 43 mL (50 mL initial test volume minus 7 mL test medium used for cell counting). Since the analytical method applied in this study was validated for 200 mL we had to combine replicates and fill up the volume to 225 mL with fresh test medium leading to a reduction in number of replicates for chemical analysis. The second challenge were the low contaminant concentration levels in our biotests. Since we used passive sampler extracts we were limited by the available extract volume and the solvent content of these when adding them to our biotest medium. To avoid solvent effects on *P. tricornutum* we could maximally spike with a solvent concentration of 0.02 % (v/v). This was equivalent to a 1:500 times dilution of the pure Speedisk extracts in the highest CT resulting in test medium concentrations in the low ng $L^{-1}$ range; concentrations that were close to realistic levels measured in grab samples (**Figure 6.3**). These concentrations were in discrepancy with the method detection limits (MDL) of the target

compounds. Anyhow, with the sometimes few quantifiable substances we were able to prove good correlation with test medium concentration predictions based on measured extract concentrations (Annex C, Figure C2). In order to keep as much information on the mixture composition as possible we decided to define the test medium concentrations by calculating based on the applied dilution of the Speedisk extracts rather than working with the actual measurements of the test medium. Following a weight of evidence approach, we nevertheless compared both, calculated and measured test medium concentrations for biotests 2 and 3 with measured grab water sample concentrations for our target compounds (**Figure 6.3**). Summed contaminant concentrations in the test medium at the highest CTs of biotest 3 were found to be factor 1.1 and 2.4 of those measured in the grab water samples for the harbor and sea samples, respectively. These values confirm that biotesting occurred at or close to realistic contaminant concentration levels.

Multivariate analysis of the target compound concentrations in Speedisk extracts of both sampling locations showed clustering amongst sampling locations and Speedisk replicates as shown in **Figure 6.4**. This indicates that we could clearly discriminate between sampling locations based on the analysis of our 88 target compounds. When comparing the Speedisk extracts within sampling locations, we observed clustering based on the extract storage time for each location, respectively. Among the measured target compounds, PCA followed by OPLS-DA identified the β-blocker atenolol as the one contributing the most to the observed variation in mixture composition due to differing extract storage time. Atenolol was detected in marine waters already in previous research [130, 138] and has been reported up to 50 ng $L^{-1}$ [130] at HZ. $EC_{50}$ (72 h) values for *P. tricornutum* have been reported at 312 mg $L^{-1}$ (95 % CIs = 262 – 371 mg $L^{-1}$). This effect concentration is several orders of magnitude above the concentrations algae were exposed to in our growth inhibition tests and can hardly be linked to any observed stimulation effects. To our knowledge, atenolol has not been tested at realistic environmental concentration levels for *P. tricornutum*. It has been tested for photosynthesis inhibition after 24 h growth with the green algae *Desmodesmus subspicatus* where it caused an activity that was clearly higher than expected from baseline toxicity.. Cleuvers (2005) reported atenolol to be non-toxic towards aquatic organisms (PNEC = 310 µg $L^{-1}$) [139]. Even though atenolol was found to explain by far the most of the observed variation in mixture composition of the effect- and no-effect causing Speedisk extracts, our single substance experiment testing atenolol has shown that on its own it cannot explain the observed stimulation effects. It is worth noting that the 88 target compounds measured in this study likely represent only a minor fraction of all contaminants present in the complex mixtures to which the algae were actually exposed. A potentially more successful application of combining biotesting and multivariate statistics for the identification of mixture effect driving substances could be as follows: in a situation where testing of Speedisk extracts from different sampling locations would result in different ecotoxicological responses (i.e. effects and no effects), the use of non-targeted chemical analysis to analyze a virtually unlimited number of substances in combination with multivariate statistics could be used to identify potential mixture effect drivers [140].

Stimulation effects in 72 h algae growth inhibition tests, as observed in our experiments, have been reported for several freshwater and marine algae in literature. Harbi et al (2017) reported slight growth stimulation of the marine green algae *Dunaliella tertiolecta* when exposed to 6.25 % and 12.5 % (v/v) of a wastewater treatment plant effluent [141]. Libralato et al. (2016)

reported growth stimulation effects ranging from 2 – 102 % on *P. tricornutum* for 75 out of 93 tested wastewater samples. The authors reported that the observed stimulation effects could not directly be linked to nitrogen (N, < 0.01 – 225 mg $L^{-1}$) and phosphorus (P, < 0.01 – 44.0 mg $L^{-1}$) concentrations that were present in all wastewater samples. Nevertheless they discussed that micronutrients such as calcium (Ca, not available, n.a.), cobalt (Co, 0.2 – 19.01 µg $L^{-1}$), iron (Fe, 2 – 28,835 µg $L^{-1}$), magnesium (Mg, n.a.), manganese (Mn, 0 – 2397 µg $L^{-1}$), potassium (K, n.a.), silica ($SiO_2$, n.a.) and sulfur (S, < 0.01 – 434 mg $L^{-1}$) might act as stimulating agents in wastewater samples [142]. It is obvious that a high availability of micronutrients might lead to an increased algal growth. However, the algal growth medium used in our experiments is composed in a way to assure exponential growth for *P. tricornutum*. The medium contains all the above listed micronutrients (N = 8.2 mg $L^{-1}$, P = 0.44 mg $L^{-1}$, Ca = 410 mg $L^{-1}$, Co = 0.41 µg $L^{-1}$, Fe = 0.035 mg $L^{-1}$, Mg = 1.3 g $L^{-1}$, Mn = 0.19 mg $L^{-1}$, K = 400 mg $L^{-1}$, $SiO_2$ = 2.0 mg $L^{-1}$, S = 840 mg $L^{-1}$) and we therefore exclude additional nutrients eventually collected with our passive samplers to be a reason for growth stimulation. This further strengthens our hypothesis that the observed stimulation effects are linked to the exposure of *P. tricornutum* to the ERCMs in our Speedisk extracts. Also, we are not aware of any studies with Speedisks reporting the binding of nutrients in literature. The occurrence of stimulation effects has been discussed in literature in detail within the context of hormetic dose-response relationships [143-146]. Calabrese and coworkers characterized the average hormetic response with a maximum stimulation of 130 – 160 % of the control value. Further, they defined the width of the low-dose stimulatory range with approximately 10-fold but observed it to be more variable in contrast to the maximum observed stimulation response. In 2 % of the plant-related cases the stimulatory dose range did exceed 1000-fold such as observed in our experiments [145, 146].

## 6.5 Conclusion

By repeating algae growth inhibition tests with Speedisk extracts over an extended time period, we observed a disappearance of stimulation effects due to, likely, chemical degradation in the passive sampler extracts. The repetition in combination with chemical analysis for a high number of PCPs, pesticides and pharmaceuticals and subsequent multivariate analysis put forward atenolol as the substance explaining the majority of the variation in mixture composition between growth-stimulating and no-effect causing Speedisk extracts. Testing of atenolol individually, however, showed no effects on the growth of *P. tricornutum*, which may suggest that non-targeted substances present in the Speedisk extracts might have been responsible for the observed stimulation effects. Further, we have shown that ERCMs can cause growth stimulation on the marine diatom species *P. tricornutum* at environmentally realistic concentrations. The identification of effect drivers in such complex mixtures remains a difficult task. Nevertheless, our findings underline the importance of testing contaminant mixtures at environmentally realistic concentration levels to increase realism in mixture toxicity testing. Further, we recommend to reduce and control the handling time and number of manipulation steps of passive sampler extracts prior to biotesting as much as possible in order to assure maintenance of the mixture composition in such complex samples. The question whether or not stimulation effects have a positive or a negative impact on *P. tricornutum* and

its surrounding in natural habitats (i.e. outside the laboratory environment), remains unsolved and requires further investigation.

While this chapter provides a novel method to combine passive sampling of ERCMs with ecotoxicity testing, the sample enrichment was limited to REF < 2. Especially for environments such as open sea locations, testing such low REF would likely only rarely result in any (negative) observed effects on most test organisms. In order to use this method in a risk assessment context, higher sample enrichment would be needed to reach effect levels in the used test organisms. To achieve this and allow the calculation of a Margin of Safety (MoS) for the different sampling locations in the BPNS, we successfully adapted the developed method in Chapter 7.

Thus, while the developed method provided a solid basis for the development of an effect-based monitoring approach, the identification of mixture toxicity driving substances using multivariate statistics was not successful. For his purpose, EDA seems to be the most promising approach [132, 147].

# 7

# A margin of safety approach for the assessment of environmentally realistic chemical mixtures in the marine environment based on combined passive sampling and ecotoxicity testing

Redrafted from:

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# 7 A margin of safety approach for the assessment of environmentally realistic chemical mixtures in the marine environment based on combined passive sampling and ecotoxicity testing

## 7.1 Introduction

Chapter 6 revealed that the developed method for combining passive sampling of environmentally realistic chemical mixtures (ERCMs) and ecotoxicity testing with *P. tricornutum* had considerable limitations in terms of enriching the ERCMs. The maximum test concentration that can be attained in a bioassay is highly dependent on biotest volume, the available sample volume, and the presence of solvent in the passive sampler (PS) extract (since a too high solvent concentration in a biotest may affect the test organisms by itself). In order to enrich the ERCMs to concentration levels that may actually inhibit the growth of *P. tricornutum* considerable method adaptations to increase the biotest sensitivity were needed. Sensitivity can be increased by selecting suitable endpoints, increasing the relative enrichment factor (REF) and/or miniaturizing the test setup [148]. For this purpose, algae growth inhibition tests can easily be adapted to testing in microplates instead of the more commonly used high-volume setups. Testing in microplates considerably reduces extract consumption and can thus be used for high throughput screening [147]. Further, working in reduced test volumes of e.g. 2 mL rather than 50 mL allows an increase of the maximum test concentration by factor 25 when adding an equal amount of passive sampler extract. Nevertheless, the conventional method for extract spiking would still be limited by the sample's solvent content. Solvent toxicity thresholds in algae growth inhibition testing with *P. tricornutum* has been reported with 0.02 % [149]. Thus in order to allow testing of higher enriched speedisk extracts, the solvent content in the extracts must be reduced.

These two adaptations, i.e. miniaturization of the biotest system and reduced solvent content in the samples, are needed to move from a priority substance-focused to an ERCM-based environmental risk assessment. This is important since it is recognised that not all substances on the Water Framework Directive (WFD) list of priority pollutants are still representative of present day contamination [23]. In addition, targeted chemical analyses on their own tend to underestimate mixture toxicity [150, 151] and risk assessments based on chemical monitoring data provide only the lower boundary of the chemical risks at any given site or moment [7]. Indeed, low concentrations or below detection limit reports of priority pollutants are not sufficient to exclude any potential ecological risks [152]. Generally, awareness is increasing that targeted chemical monitoring and priority substance driven research alone cannot account for the complexity of chemical mixtures present in most aquatic environments [153]. Our limited knowledge on the complex chemical mixtures that many aquatic organisms are exposed to and eventually affected by foster the need for effect-based rather than chemical analysis-driven monitoring and effect assessment tools.

In this research, we adapted the previously developed effect-based monitoring method to derive a Margin of Safety (MoS) of ERCMs in the marine environment and applied it in a case study to the Belgian Part of the North Sea (BPNS). The method consists of passive sampling of an ERCM, followed by sampler extraction, and biotesting (here: the diatom *P. tricornutum*) of a range of dilutions of this (enriched) extract, with each dilution representing an enrichment

factor in terms of chemical concentrations relative to the actual aquatic concentrations in the environment. The MoS is defined as the highest REF with no significant reduction of diatom growth rate compared to a control.

## 7.2 Materials and methods

### *7.2.1 Passive sampling and sampler treatment*

The methods presented in this chapter, were only applied to samples from sampling campaign 2 (SC2), SC3 and SC5. Time between sampler recovery and extraction was 19 months (SC2), 15 months (SC3) and 4 months (SC5). Based on the findings in chapter 6, all samples were tested within less than 2 weeks of extraction and chemical analysis occurred no later than 1 month after extraction.

Speedisks were rinsed and extracted with 10 mL HPLC grade methanol : acetonitrile (1:1, v/v) as described in Chapter 3. In addition to the triplicate speedisks per sampling location, three speedisks deployed at SC3 HO were extracted and extracts split into two equal parts of 4.9 mL each, while all other extracts were kept undivided before full evaporation of the extraction solvent under a gentle nitrogen stream at 25 °C. The separation of SC3 HO extracts occurred in order to compare the previously developed extraction procedure (see Chapter 6) with the adapted method described here. Finally, the precipitate of half of the split SC3 HO extracts was reconstituted in 490 µL of HPLC methanol : HPLC water (10:90, v/v) acidified with 0.1 % formic acid and 0.01 % $Na_2EDTA.2H_2O$ (further called “MeOH extracts”) while the other half was reconstituted in 490 µL HPLC water (further called “$H_2O$ extracts”). For all other samples, the precipitate was reconstituted in 1 mL HPLC water. Reconstituted extracts were ultra-sonicated for 1 min, vortexed for 20 s and centrifuged at 3,000 rpm for 5 min. Finally, the liquid phase was transferred to a HPLC vial and stored at – 20 °C until instrumental analysis and biotesting.

Prior to biotesting, concentration series of the passive sampler extracts were prepared by diluting the extracts along a 1:3 dilution series. This dilution factor was chosen to account for the limited sample volume available on the one hand and to allow a maximum REF on the other hand. For this purpose, 500 µL original extract was diluted by adding 9.5 mL growth medium [67].This growth medium was prepared in a way to account for dilution via spiking with speedisk extracts. Therefore, salts and vitamins were added at 105 % of the concentrations described in the guideline [67]. For the following concentration treatment (CT), 3.5 mL of this solution were diluted by adding 7 mL non-adjusted growth medium. This was repeated 6 more times to obtain a serial dilution with a total number of 8 CTs per speedisk extract. The extract concentrations are expressed as sum-analyte concentrations throughout this chapter.

### *7.2.2 Chemical analysis*

Based on previous experience [149] speedisk extracts were analyzed and tested within maximum four weeks of extraction. Chapter 6 has shown that it is better to predict biotest concentrations based on those concentrations determined in speedisk extracts in order to maintain a maximum of available information since concentrations in microplate wells conflict

with analytical detection limits [149]. All chemical analysis was performed as described in Chapter 3.

### *7.2.3 Algae growth inhibition testing*

All biotests were performed using biotest system 4 (see Chapter 2). Biotest concentrations were expressed as REFs ($REF_{geomean}$) of all measured substances calculated as geometric mean of individual REF per substance ($REF_i$). The $REF_i$ was calculated in function of the analyte concentrations measured in speedisk extracts and grab samples (see annex D, Table D1) taken at the respective sampling location during sampler deployment and retrieval (mean of sampler deployment and retrieval) where REF = 1 corresponds to environmental concentrations ($C_w$). This has been applied in previous research (see Chapter 6) and is further described by ***(Eq. 7.1)*** [154], where d indicates the applied dilution factor of the treatment in the biotest. Substances with a measured concentration in blank speedisks were excluded from the dataset and not accounted for in all REF calculations. REFs of the individual compounds were calculated as follows:

$$REF_i = \frac{\frac{C_{i,extract}}{\frac{(C_{i,grab\ sample\ deployment} + C_{i,grab\ sample\ retrieval})}{2}}}{d} \quad \textbf{(Eq. 7.1)}$$

Then, the REF of the ERCM (as present in the Speedisk extract) was determined as the geometric mean ($REF_{geomean}$) of the individual $REF_i$ across all measured substances:

$$REF_{geomean} = \sqrt[n]{\prod_{i=1}^{n} REF_i} \quad \textbf{(Eq. 7.2)}$$

Substances with concentrations below the grab sample method detection limit (MDL) were not included in the calculation of the REF. An overview of the $REF_i$ per sampling location and campaign is given in annex D (Table D2).

### *7.2.4 Data analysis*

Finally, fluorescence measurements were converted to cell counts using a calibration series (see Annex D, Table D3) and diatom growth rates in all CTs were calculated ***(Eq. 2.1)*** and statistically compared with those in the control using one-way ANOVA, followed by Dunnett's multiple comparisons test (α = 0.05). The $NOEC_{REF}$ (NOEC expressed as $REF_{geomean}$) was defined as the highest $REF_{geomean}$ with no significant reduction of diatom growth rate compared to a control. The $NOEC_{REF}$ is also equal to the MoS for this diatom species. The MoS was determined for each sampling location based on growth inhibition of *P. tricornutum* exposed to various dilutions of speedisk extracts.

Correlation analysis comparing the contaminant concentrations in MeOH extracts and $H_2O$ extracts was performed using Pearson correlation (α = 0.05) including the triplicate data for both extraction methods. All statistical analyses of the biotest results and the chemical analysis were conducted using GraphPad Prism version 5.01 for Windows [133].

### *7.2.1 (Sparse) principal component analysis*

Using enriched speedisk extracts led to a number of samples exerting high toxicity on algae growth (see results section) providing a set of samples with a high variability in terms of response. Since we have shown before (see Chapter 6) that this variability in response is most likely a result of differing chemical concentrations in speedisk extracts, we explored our data using principal component analysis (PCA) to find clusters inherent to the chemical concentrations in the different speedisk extracts and CTs. For each sample, the chemical concentrations measured for each of the 89 target PCPs, pesticides and pharmaceuticals were used (annex D, Table-D4-D6) as input data. Per speedisk extract we included all CTs exerting statistically significant growth inhibition plus the highest CT not exerting growth inhibition (annex D, Table D7).

PCA has one obvious drawback, being the fact that the loadings of all principal components (PCs) are typically non-zero [155]. This makes it difficult to interpret the derived PCs. While a dimensionality reduction is promising to improve PC interpretation, sparse PCA also allows to reduce the number of explicitly used variables [155]. Sparse PCA was performed including the first 9 PCs from the PCA. We enforced sparsity via the argument ***sparse*** = *"varnum"* and fixed the number of non-zero components to 10 for each of the 9 PCs. All multivariate analysis was performed in R Studio [83] using the 'drc' (PCA) [82] and 'elasticnet' (sparse PCA) packages [155].

## 7.3 Results

### *7.3.1 Comparison of extraction methods*

As described before, three speedisks deployed at SC3 HO were extracted and split into two parts and reconstituted in either HPLC methanol : HPLC water (10:90, v/v) acidified with 0.1 % formic acid and 0.01 % $Na_2EDTA.2H_2O$ (MeOH extracts) or HPLC water ($H_2O$ extracts) to test whether the different reconstitution solvents had an impact on the mixture composition of the speedisk extracts. **Figure 7.1** shows the results of the chemical analysis of 89 target substances as a correlation plot of the contaminant concentrations in the MeOH extracts (x-axis) vs. the contaminant concentrations in the $H_2O$ extracts. Correlation analysis of the averaged concentration for 39 substance pairs resulted in a significantly positive ($p < 0.0001$, $r^2 = 0.9691$) correlation of the contaminant concentrations in the different extracts. Besides the 39 substances detected in both extracts, four were detected in only one: propylparaben was detected in all three replicates and ketoprofen in one replicate of the MeOH extracts, while alprazolam was detected in two and lamivudine in one replicate of the $H_2O$ extracts. The remaining 46 compounds were not detected in any of the extracts. For those substances detected in both extracts, 88 % had a concentration within less than 2 fold (96 % within less than 3-fold).

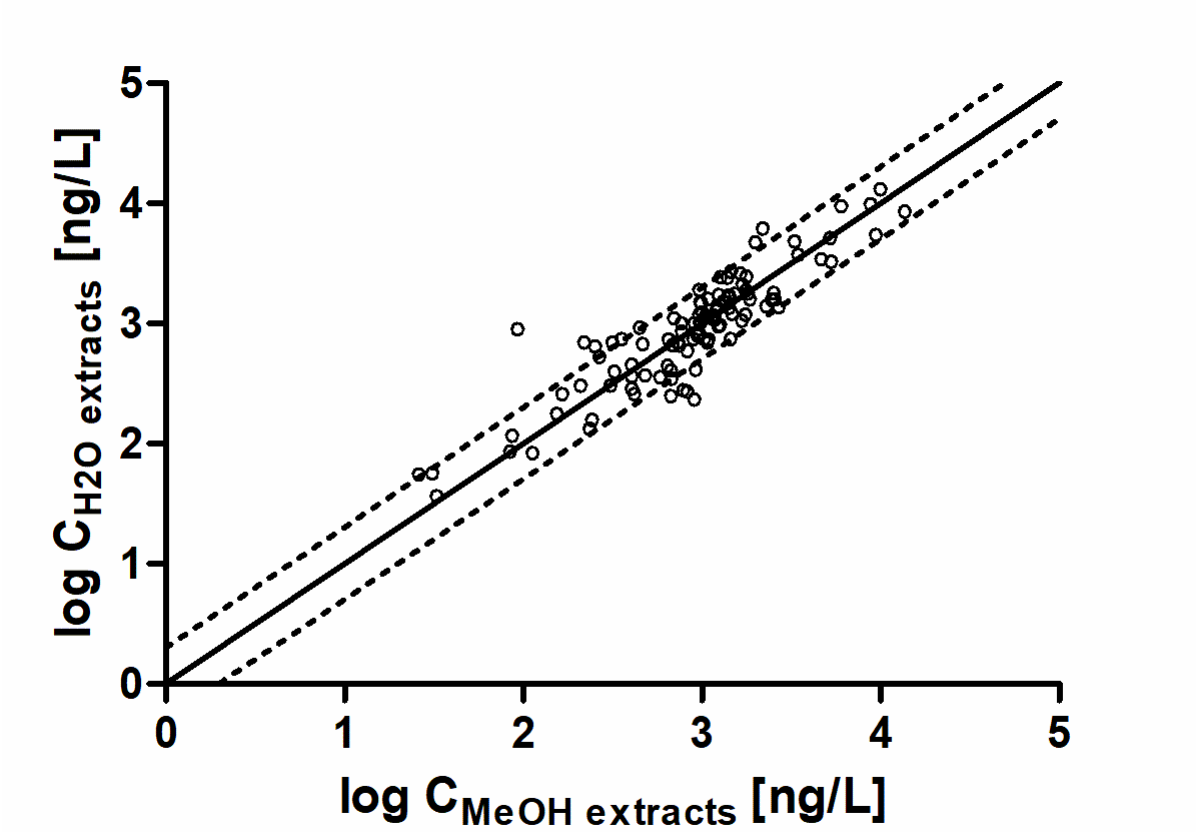


**Figure 7.1 Correlation plot comparing the contaminant concentrations measured in the MeOH-extracts (x-axis) and the $H_2O$-extracts (y-axis). The solid line shows the 1:1 trendline and dashed lines indicate the 2-fold line.**

### *7.3.2 Marine speedisk extract testing*

A detailed overview of the measured pH in the three biotests is given in annex D (Table D8). Overall, the pH varied 0.2 (7.9 – 8.1, SC2), 0.7 (7.6 - 8.3, SC3) and 0.6 (7.6 – 8.2, SC5) units during the tests. The temperature varied by 1.8 °C (21.0 °C – 22.8 °C, SC2), 2.0 °C (21.1 °C – 23.1 °C, SC3) and 2.0 °C (20.8 °C – 22.8 °C, SC5) throughout the test period. Both pH and temperature variation thus fulfilled the recommended validity criteria for 72 h growth inhibition testing with *P. tricornutum* [69]. A summary of the results of all algal growth inhibition tests with speedisk extracts is shown in **Figure 7.2**.

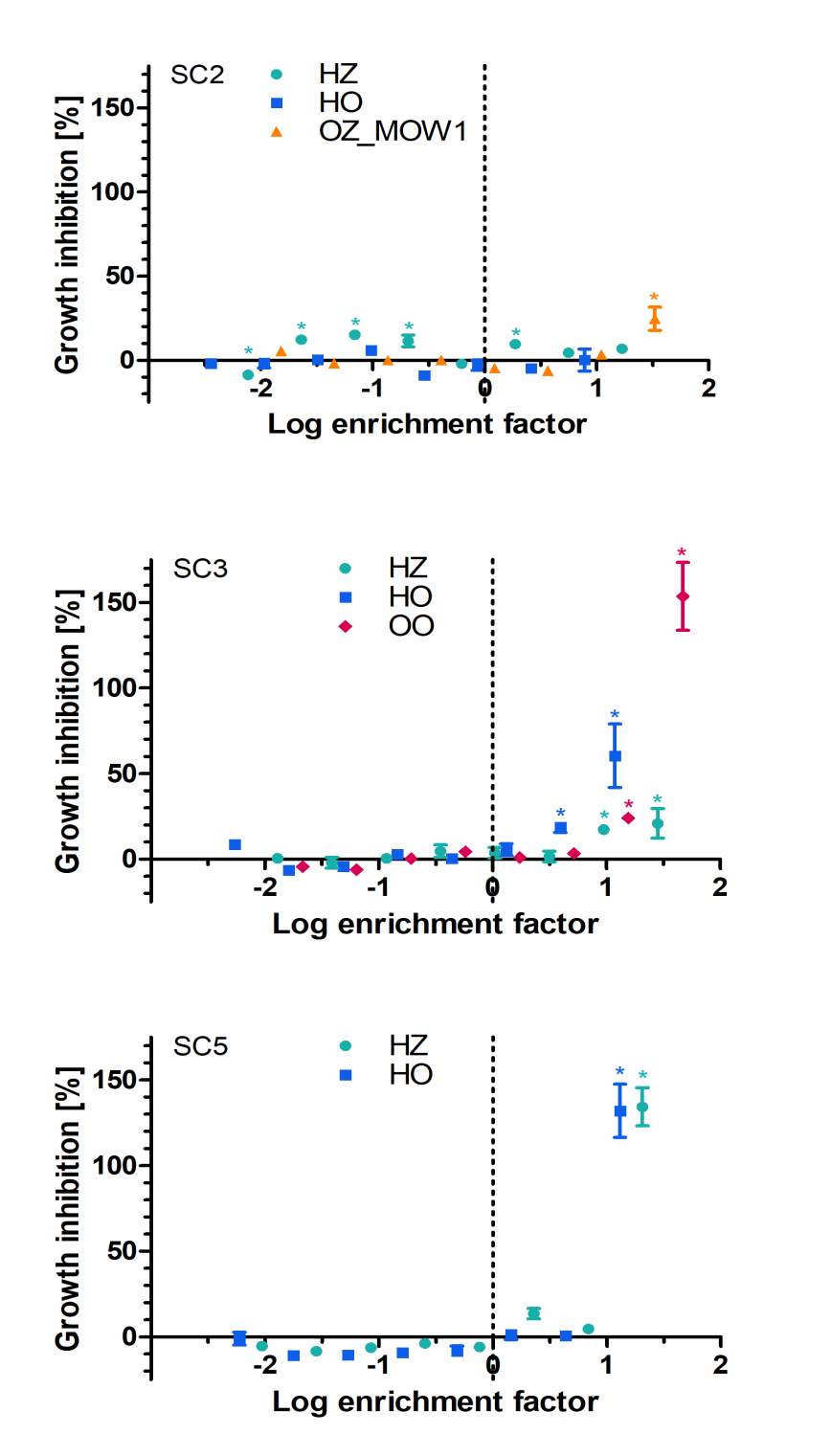


**Figure 7.2 Summary of speedisk extract testing for sampling campaigns 2, 3 and 5. Shown are the percentage growth inhibition vs. the log relative enrichment factor for speedisk extracts from the different sampling locations HZ (Harbor Zeebrugge), HO (Harbor Ostend), OZ_MOW1 (coastal sampling location near Zeebrugge) and OO_X (coastal sampling location near Ostend). The vertical dashed line indicates the summed contaminant concentrations in corresponding grab samples representing realistic environmental concentration levels. Data shows the average growth inhibition of triplicate speedisks for each location. Error bars represent the standard error of the mean and data marked with an asterisk showed significant growth inhibition as compared to the control treatments.**

### *7.3.3 Link to environmental concentrations and margin of safety determination*

Based on grab water samples taken at the start and end of the passive sampler deployment period, the average contaminant concentrations at each sampling location have been determined. A comparison with the sum analyte concentrations in the speedisk extracts allowed the definition of REFs ***(Eq. 7.1)***. The measured contaminant concentrations per extract and in the grab samples can be consulted in annex D (Tables D4 – D6). REFs tested ranged from 0.0035 (lowest test concentration) to 33 (highest test concentration) for SC2, from 0.0043 to 44 for SC3 and from 0.0058 to 19 for SC5. In general, the two to three highest CTs represented enriched environmental mixtures while the lower five to six treatments were dilutions of realistic environmental mixtures. The third highest CT approximately represented realistic seawater levels as indicated by the vertical dashed line in **Figure 7.2**.

Margins of safety could be determined for all four sampling locations as shown in **Table 7.1**. Yet, analysis of SC2 HZ resulted in no concentration-dependent effects and no MoS could be determined. Speedisk extracts for SC2 HO did not exert any effects on algal growth resulting in the highest REF to define the MoS. For SC3 and SC5 the MoS ranged from 1.1 to 6.5 and statistically significant effects (one-way ANOVA followed by Dunnett's multiple comparisons test, $\alpha = 0.05$) on the growth of *P. tricornutum* were measured as of REF = 3.2.

**Table 7.1 Summary of algae growth inhibition testing of speedisk extracts and link to environmental concentrations. Shown are the highest relative enrichment factors (REF) resulting in no statistically significant effect (= MoS) and the lowest REF resulting in a statistically significant effect on the growth of *Phaeodactylum tricornutum*. The margin of safety is given as the highest REF resulting in no-observed effect on algal growth. No results were determined (ND) for SC2 HZ because effects were not concentration-dependent. For SC2 HO no effects were observed and thus defining a lowest effect REF was not applicable (NA).**

| Sampling campaign (SC) & location | Margin of safety = Highest no-effect REF (% growth inhibition) | Sum analyte C (µg $L^{-1}$) MoS | Lowest effect REF (% growth inhibition) | Sum analyte C (µg $L^{-1}$) lowest effect REF |
|---|---|---|---|---|
| SC2 HZ | ND | ND | ND | ND |
| SC2 HO | ≥8.1 (0.18) | 4.7 | >8.1 | NA |
| SC2 SZ | 11 (3.7) | 1.5 | 33 (25) | 4.4 |
| SC3 HZ | 2.8 (1.5) | 0.22 | 8.3 (17) | 0.67 |
| SC3 HO | 1.1 (5.6) | 0.21 | 3.2 (19) | 0.63 |
| SC3 SO | 4.9 (3.3) | 0.26 | 15 (24) | 0.78 |
| SC5 HZ | 6.5 (4.7) | 0.60 | 19 (134) | 1.8 |
| SC5 HO | 4.3 (0.51) | 2.2 | 13 (132) | 6.7 |

### *7.3.4 (Sparse) principal component analysis*

PCA resulted in no obvious clustering in relation with effects on algal growth. ***Figure 7.3*** shows the Biplot of the first two PCs of the PCA. These two principal components (PCs) explained 63 % of the variability in the chemical concentration data. The cluster of samples on the left of the loadings arrows indicate a lower concentration of most chemicals in these samples as compared to the average concentration in all samples. Among the relatively diffuse distribution of the samples in the biplot, some groups show clustering according to the combination of SC and location such as e.g. samples 1,2 and 3 representing samples from SC2 HO that did not

cause any effect on the growth of *P. tricornutum*, samples 43, 45 and 47 representing the replicates of SC5 HO with statistically significant growth inhibition or samples 44,46 and 48 representing samples from SC5 HO not affecting algae growth. The screeplot (annex D, Figure D1) shows the cumulative variance of the first 15 PCs suggesting that > 90 % of the overall variance was explained by the first 9 PCs.

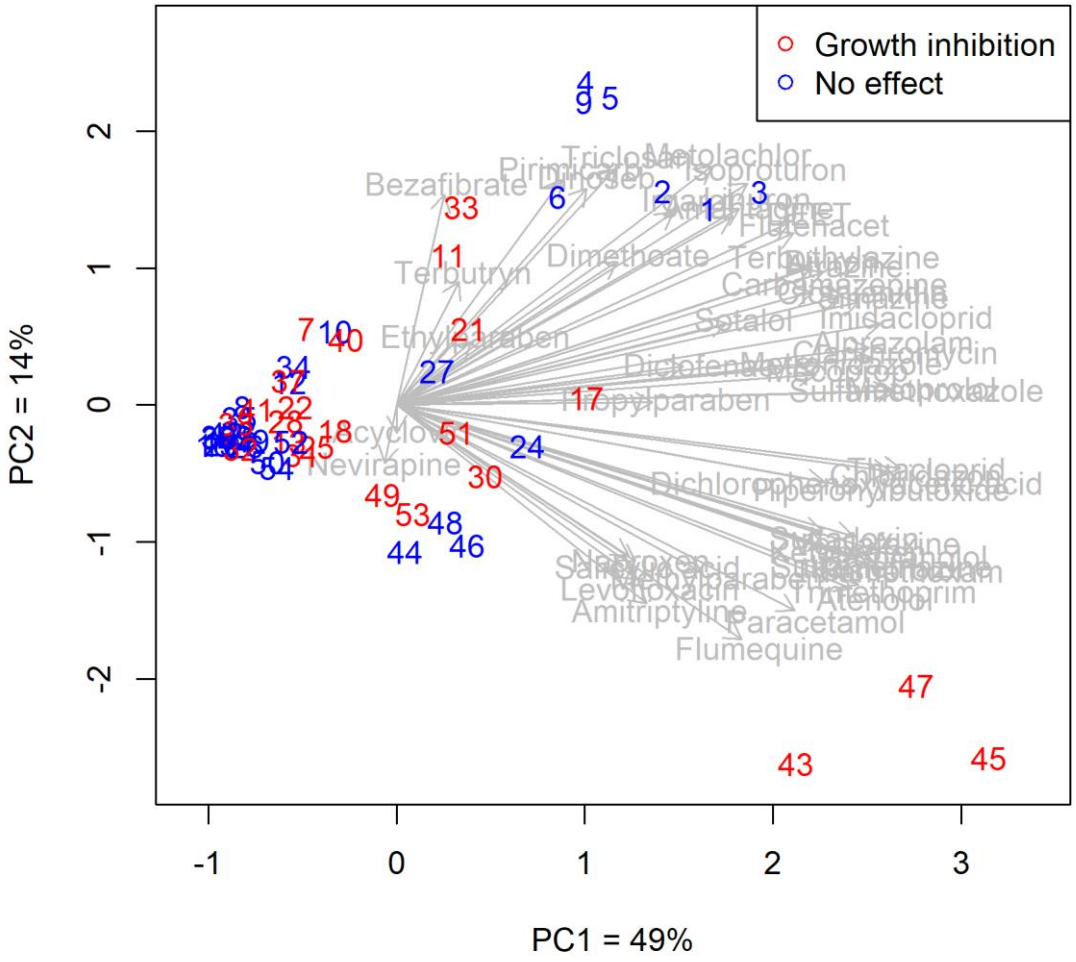


**Figure 7.3 Biplot of the principal component analysis of speedisk extract concentrations of 89 target PCPs, pesticides and pharmaceuticals. Numbers refer to Table D7 in annex D and indicate the different samples tested in algae growth inhibition tests. The grey arrows show the loadings of the principal components.**

The sparse PCA clearly reduced the complexity of the loadings and allowed an easier interpretation of the PCA. ***Figure 7.4*** shows the results of the sparse PCA. A look at the loadings of the PCs shown in annex D (Table D9) indicates that a 55 % of the target substances have a loading of 0 for all 9 PCs meaning that they are uninformative. Thus, these 29 substances do not contribute to the variability in the dataset. Sodium diclofenac was negatively correlated with PC1 and positively correlated with PC2 whereas sotalol showed negative correlation with both PC1 and PC2. Carbamazepine was negatively correlated with PC1 but had no impact on PC2, and the other way around for naproxen. Samples 33, 37 (both SC3 OO_X exerting growth inhibition), 43 and 45 (both SC5 HO exerting growth inhibition) were negatively correlated with

PC1 and slightly positively correlated with PC2. Sample 47 (SC5 HO exerting growth inhibition) was strongly negatively correlated with PC1. Most samples exerting no toxicity either showed no correlation with the first two PCs or a slightly to moderately negative correlation with PC1 but generally no correlation with PC2.

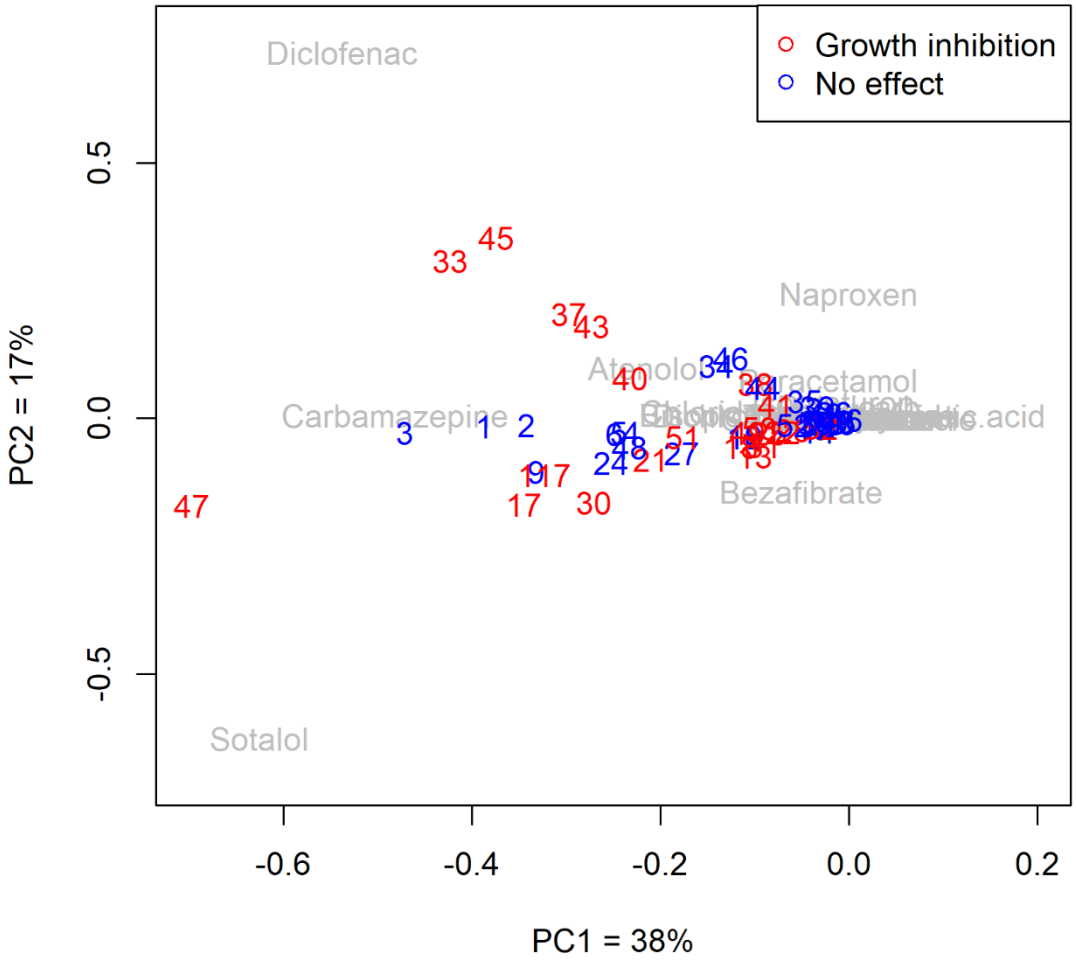


**Figure 7.4 Sparse principal component analysis of speedisk extract concentrations of 89 target PCPs, pesticides and pharmaceuticals. Numbers refer to Table D7 in annex D and indicate the different samples tested in algae growth inhibition tests. The substance names show all chemicals with non-zero loadings for the 9 included principal components.**

## 7.4 Discussion

In a preliminary test we compared the contaminant mixtures in speedisk extracts reconstituted in two different solvents (MeOH and $H_2O$ extracts). Our results show that there was a positive correlation between chemical concentrations in the extracts obtained from the two reconstitution methods. The concentrations for 88 % of the detected substance pairs was found to be within a factor 2. When exposing the marine diatom *P. tricornutum* to speedisk extracts, we observed significant effects on its growth in treatments with REF ≥ 3.2. Based on these results, we defined MoS for all four sampling locations in the BPNS ranging from 1.1 to 11. From a conventional risk assessment point of view, there would typically be a need for applying an assessment factor (AF) of minimum 10 to the lowest of available species NOECs to account for lab to field extrapolations [33]. Thus, 5 of the 8 samples studied here would, according to conventional risk assessment, be considered at risk, even if chronic NOECs for fish and invertebrates would be higher than that of the diatom (i.e. even if diatoms were the most sensitive species).

There appears to be some differences between SCs, with MoS between ≥ 8.1 – 11 (SC2), between 1.1 – 4.9 (SC3), and between 4.3 – 6.5 (SC5). There is no obvious difference of the MoS in coastal locations (4.9 - 11) compared with harbor locations (1.1 – ≥ 8.1), but sample size is too small for a definitive conclusion. In addition, there is considerable uncertainty in the derived MoS, due to the fact that it is derived from a NOEC estimation from tests with a relatively large spacing factor between two CTs (~factor 3). Indeed, a REF > 13 always resulted in growth inhibition, while a REF ≤ 2.8 never resulted in growth inhibition, but REF values in-between were associated with cases of both growth inhibition ($LOEC_{REF}$) and no growth inhibition ($NOEC_{REF}$). To decrease this uncertainty in MoS in the future, we recommend to focus on testing dilution series of Speedisk extracts with REF > 1, and with a spacing factor ≤ 2. This approach would both reduce the sample consumption per CT and decrease the uncertainty associated with the MoS being based on the $NOEC_{REF}$. In addition, with more dilutions being tested over a narrower range of REF, it could be considered to derive an $EC_{10}$ expressed as REF using dose-response analysis and defining the MoS = $EC_{10,REF}$. These optimizations could make this approach be more efficient, less resource-demanding, and generate more reliable assessments.

### *7.4.1 Combining passive sampling and biotesting*

Combining passive sampling and biotesting has been applied and confirmed to be highly suitable to identify the ecotoxicological relevance of complex contaminant mixtures [36]. Similar to lowering the detection limit in analytical chemistry, there is a need for sample enrichment for biological effects detection. Indeed, the fact that all lowest effect REF values were >1 (**Table 7.1**), indicates that detecting significant effects in marine samples without enrichment is unlikely, at least for the diatom tested here. In the context of combining passive sampling and biotesting this is achieved by increasing the maximum REF that can be tested. Increasing the REF can be challenging depending on factors such as e.g. sample volume, biotest volume, and solvent used for the SPE elution step. Usually passive sampler extracts are dosed using solvents. This requires a REF of 10,000 – 100,000 in the extracts due to the maximum acceptable solvent concentrations (usually 0.1 – 1 %, v/v) in biotests to reach a REF of 100 depending on the solvent acceptability in the biotest system [147]. Such high REF are easily reached with large volume SPE (LV-SPE) [156] but rather unrealistic for passive

sampling. LV-SPE is a relatively novel time-integrated approach for water sampling. It offers the possibility to sequentially extract large water volumes (50 – 1000 L) with different adsorbents and can thus target a broad range of water contaminants [147]. It is a more or less exhaustive water extraction procedure that does not require compound-specific calibration [147]. As such, it could be an interesting method to apply for large volume sampling of seawater since it is a rapid method allowing for sample conservation on a sorbent of choice.

Here, we investigated the possibility to reconstitute passive sampler extracts in HPLC-water instead of in an organic solvent (mixture). Chemical analysis showed a statistically significant positive correlation of the mixture compositions in MeOH and $H_2O$ extracts, with 88 % of the substance's concentrations within less than a factor 2. Reconstitution in HPLC-water was thus suitable to overcome solvent-related challenges while allowing REFs up to 44 in the biotest medium. Therefore, the reconstitution in HPLC water was used as the standard method for all biotests performed in this research.

While being a useful screening tool for realistic mixtures, testing passive sampler extracts is associated with one major restriction: each passive sampler has a specific binding capacity related to substance polarities. Kim Tiam et al (2016) stated that passive sampler extracts do not reveal the entire complexity of the studied water body, since each sampler has a defined selectivity in terms of polarity or charge. Most of the commonly used passive samplers have affinity ranges spanning about 3 - 4 log $K_{OW}$ units [24]. For the "pharmaceutical" configuration of Polar Organic Chemical Integrative Sampler (POCIS) [157] this affinity is in the range of log $K_{OW}$ 1 – 4 while styrene-divinylbenzene (SDB) Empore® disks embedded in Chemcatcher have shown to sample substances with a log $K_{OW}$ 0 – 4 [158]. Equilibrium passive samplers based on e.g. polydimethylsiloxane (PDMS) silicone rubber (SR) as the receiving phase or semi-permeable membrane devices (SPMD) can be used to sample compounds in the hydrophobicity range of log $K_{OW}$ 3 – 10 [24]. Data from Huysman et al. (2019) has shown that $H_2O$-philic divinylbenzene speedisks are able to sample a broad polarity range spanning from log $K_{OW}$ -0.13 – 9.85 and thus including both very polar and non-polar compounds. Consequently, speedisks containing this sorbent considerably broaden the sampled polarity range as compared to commonly used passive sampling devices and their extracts are very likely to represent a relevant mixture of contaminants. When reconstituting these mixtures in water, one might expect that only the polar fraction of the mixture would be maintained in the sample. Nevertheless, a correlation analysis between log $K_{OW}$ and $REF_i$ did not support this expectation, at least for the polarity range from log $K_{OW}$ -0.13 (TMX) to 4.9 (amitriptyline) as shown in annex D (Figure D2). For substances with a log $K_{OW}$ above this range it will become increasingly unlikely that they can be dissolved in HPLC water, but this requires additional research.

### *7.4.2* Environmental realism in algae growth inhibition testing with passive *sampler extracts*

Diatoms account for >20 % of the photosynthesis occurring in global oceans [159] and play a crucial role at the basis of the oceanic food web [160]. In this study exposure of *P. tricornutum* to enriched passive sampler extracts resulted in significant growth inhibition (17 – 134 %) as of 3.2-fold enrichment of realistic environmental mixtures. Similar observations have been reported before in other studies. Shaw et al. (2009) observed a significant yield inhibition in *P. tricornutum* exposed to SDB-reverse phase sulfonate (RPS) Empore disk extracts deployed

at a river mouth in the Great Barrier Reef, Queensland, Australia. Similar to this study they observed about 25 – 50 % inhibition at REF 5 – 10 [36]. In an effect-based and chemical identification monitoring program of organic pollutants in European surface waters, Tousova et al. (2017) reported algae growth inhibition $EC_{50}$ values for the green algae *Raphidocelis subcapitata* to occur at REF ≥ 17 and LOEC values at REF ≥ 11 [156]. Their LOEC values are in good agreement with our findings, except for two of our samples (SC3 HZ and SC3 HO), where REF at LOEC = 8.3 and 3.2, respectively. Due to the limited number of tested dilutions that actually exerted significant effects (maximum 2 per test), we could not reliably determine $EC_{50}$ values. For those tests that showed growth inhibition ≥ 132 %, the $EC_{50}$ values would likely be in the range 4.3 < REF < 44. This would situate some of our $EC_{50}$ values in a range that is lower than the lowest reported by Tousova et al. (2017).

In another study investigating effects of trace levels of complex mixtures on oceanic phytoplankton, Echeveste et al. (2010) observed lethal effects on wild marine phytoplankton communities [160]. These observations suggest an effect that goes beyond growth inhibition and thus being an irreversible reduction of phytoplankton biomass and production. Similar effects were observed in our study for SC5 HZ and SC5 HO extracts where exposure to REF ≥ 13 led to a decrease of the initial cell number, suggesting cell death (as indicated in Figure 3 by growth inhibition values exceeding 100 %). Echeveste et al. (2010) concluded that levels of pollution reaching oceanic waters are approaching concentrations that significantly affect oceanic phytoplankton due to the complex cocktail formed by a mixture of a huge variety of chemicals [160].

### *7.4.3 Seasonal trends and seasonality effect(s)*

While grab sample data generally provides total measured concentrations and does not distinguish between bioavailable and non-bioavailable fractions of chemicals, passive sampling does [122]. Indeed, the 89 target compounds analyzed in speedisk extracts were mainly polar organic chemicals with expectedly low sorption to organic matter or biota. We are also aware that there was certainly uptake of a considerable fraction of non-polar substances like e.g. steroids or phthalates [108]. Unfortunately, the analytical method developed for the latter substance groups was not compatible with the here developed approaches. This was due to addition of deuterated internal standards for analytical purposes prior to Speedisk extraction. The latter was not part of the analytical method developed for the analysis of PCPs, pesticides and pharmaceuticals [71] where it would have been problematic because of an introduction of non-naturally occurring chemicals to the samples biotests were spiked with.

From a consumer perspective, especially the use of pesticides and personal care products like e.g. UV blockers is subject to high fluctuations. As an example, O'Brien et al. found a strong correlation of pesticide concentrations in an Australian estuary and harvesting season or rainfall events during a 2-year monitoring campaign with monthly sampling [161]. Our monitoring was performed twice a year and determination of such trends would require a higher sampling frequency. Nevertheless, the higher effects observed in tests with samples from SC3 and SC5 as compared to samples from SC2 may be associated with an increased pesticide use. For future monitoring a higher sampling frequency would be advised. Combined with e.g. pesticide and PCP use information along the Belgian coast and along the main input rivers could help to understand the chemical input routes into the BPNS.

With regards to potential seasonality effects, the following should also be considered: SC2 occurred mainly from December-January while SC3 and SC5 represent samples from April-June and April-May, respectively. It can be expected that between May and July (SC3 and SC5) the sedimentation flux of organic matter is reduced due to slow sinking of (dino)flagellates. Based on equilibrium partitioning, persistent organic pollutants (POPs) such as e.g. PCBs are expected to be released back to the water phase at that time of the year [162]. When entering (marine) waters POPs are very likely to sorb to organic matter. Their environmental fate is strongly associated with biogeochemical processes and equilibrium-driven between different compartments. Especially in the marine environment, their fate is strongly associated with phytoplankton biomass dynamics. Phytoplankton concentrations usually peak in April and September and reach their minimum in December/January. This suggests that POPs are usually present in marine waters at higher concentrations in April/May (SC3 and SC5) than in December/January (SC2) [162]. These findings, together with those of Claessens et al (2015) suggest that the observed effects may be associated with the presence of POPs rather than CECs in the speedisk extracts [130]. This is further supported by the relatively low toxicity observed for 19 out of 23 CECs (with exception of the relatively high toxicity of neonicotinoid insecticide on *N. spinipes*) when tested individually (see Chapter 4).Ecotoxicity studies in the Belgian Part of the North Sea

In previous studies with SR sheet passive samplers deployed in the same harbors as sampled in this study, Claessens et al. (2015) and Everaert et al. (2016) investigated effects of realistic mixtures of mainly non-polar contaminants on the growth of *P. tricornutum*. In these studies, the authors applied equilibrium-based partitioning (passive dosing) to spike algae test medium resulting in REFs of 1 under equilibrium conditions. Here, Claessens et al. (2015) observed growth inhibition on *P. tricornutum* neonicotinoid insecticide when exposed to passive samplers deployed in three SCs between April 2008 and October 2009 at HZ and HO [130]. This suggests that the non-polar fraction of chemicals could significantly contribute to the toxicity of ERCMs on marine diatoms rather than the polar fraction of chemicals.

### *7.4.4 Margin of safety approach – Possibilities & Limitations*

Overall the MoS approach allowed the definition of a “safety range” for ERCMs in the BPNS. It provides a methodological concept for an effect-based monitoring approach that, with a few adaptations, could be transformed into a regulatory useful effects-based method for mixture-based risk assessments. Below, we list possibilities and limitations.

#### *7.4.4.1 Requirements for risk assessment*

While our method was developed for testing with the marine diatom *P. tricornutum*, the basis for environmental risk assessment typically requires endpoints for at least 3 species representing three trophic levels [33]. Thus, in order to fulfill the requirements for the basic set of endpoints, our method would need to be adapted for the use with e.g. the 7-day larval development test with *Nitocra spinipes* and the fish early life-stage toxicity test.

#### *7.4.4.2 Limited enrichment of extracts*

In most cases enriching ERCMs is needed in order to reach effect thresholds in various bioassays [36]. Most passive sampler extracts are spiked using a carrier solvent, therewith by default limiting the maximum extract concentration to maximally 0.1 – 1 % in biotests [147].

The method applied here is not limited by any solvent since extracts are reconstituted in HPLC water. Nevertheless, limits are defined by the extract volume (1 mL) and the biotest volume (2 mL). For regulatory purposes testing a range of REF 1 – 10 would be ideal since i) testing of this small REF range reduces the spacing factor between each REF, whilst the number of tested REF's can remain the same, ii) when considering the typical AF of 10 for lab to field extrapolation, observing no effects up to REF 10 can be considered equivalent to "no risks", and iii) testing this reduced concentration range is associated with reduced work load and costs.

#### *7.4.4.3 Calculation of the REF*

The calculation of the $REF_{geomean}$ is based on the individual $REF_i$ of the measured contaminants. This is associated with one important limitation: targeted chemical analysis takes into account only a pre-defined set of compounds and does not give any information about other substances present in the sampled mixtures. On the other hand, adsorption based samplers such as the speedisks provide excellent enrichment of specific analytes [148] and can easily be linked to chemical analysis [149].

### *7.4.5 (Sparse) principal component analysis*

The screeplot of the PCA indicated that the first 9 PCs explained 90 % of the overall variance in the data. Yet, the interpretation of the PCA biplot was difficult due to a relatively wide distribution of the loadings (chemicals) and no clear clustering of the samples affecting the growth of *P. tricornutum* or those not exerting any effect. Nevertheless, for a few groups of samples there was clustering in function of SC and location, indicating relatively similar sample composition in terms of concentrations of the target substances in these extracts. The biplot of the sparse PCA suggests that sodium diclofenac is associated with growth inhibition effects observed in samples 33, 37 (both SC3 OO_X), 43 and 45 (both SC HO). Both sodium diclofenac and naproxen showed a positive correlation with PC2 while a positive correlation with PC2 was associated with growth inhibition for 5 out of 7 samples. Sodium diclofenac and naproxen have also been identified as substances with a median RQ > 1 in our screening-level risk assessment (see Chapter 5) even though algae were not found to be the most sensitive species group in both cases. Also, individual substance testing with *P. tricornutum* (Chapter 4) for sodium diclofenac resulted in no effects at concentrations up to 100 mg $L^{-1}$. Naproxen has not been tested within this thesis.

While PCA has been shown to evaluate similarities in composition between chemical mixtures [163], in our case, interpretation of the biplot was rather difficult due to broad scattering of the loadings. The use of sparse PCA allowed a reduction of the complexity and a more sparse representation of the data. As suggested by Eide et al. (2004) PCA offers a valuable tool for data exploration and a pre-selection for sample prioritization. In our case, we applied it with the aim of prioritizing mixture toxicity driving chemicals. Still, the targeted approach including only 89 PCPs, pesticides and pharmaceuticals in our analysis seemed to be a limiting factor and should be extended with non-target data for future applications. Extended with OPLS-DA this method may then be used for chemical prioritization purposes [164] which in the case of mixture testing translates into toxicity driver identification.

## 7.5 Conclusion

This chapter presents a novel effect-based method to derive a MoS for ERCMs in the marine environment based on passive sampling. $H_2O$-philic divinylbenzene, the sorbent included in the speedisk passive samplers, has been shown to sample a broad polarity range and reconstitution of the Speedisk extracts in HPLC water is a promising approach when combined with biotesting as it allows higher REFs to be tested. Samples with a REF ≥ 13 have always been shown to significantly affect the growth of *P. tricornutum* and MoS were found to be in the range of 1.1 to 11 across four sampling locations (and three SCs) in the BPNS. For 5 out of 8 samples the MoS was found to be lower than 10 which represents the typically lowest possible AF applied to no effects ecotoxicological data in conventional environmental risk assessment, suggesting ecological risks for these sampling locations. In conclusion, we have taken a first important step in the development of a ready-to-use method for effect-based monitoring and risk assessment of ERCMs, which we here explored with the marine diatom *P. tricornutum* as test species. However, in order to make this methodology fully compliant with conventional risk assessment it would be required to extend the ecotoxicity test battery. We would recommend the adaptation of the method to at least one chronic test with a marine crustacean such as e.g. the larval development test with *N. spinipes* and one (sub)chronic test with fish such as e.g. the fish early-life stage toxicity test with *Cyprinodon variegatus*. In that case, and if it can be argued that (sub)chronic testing with 3 sensitive species from three trophic levels is sufficient to allow for an AF = 10, the MoS values could be translated into a conventional RCR of the ERCM, as follows:

$$RCR = \frac{10}{Minimum\ (MoS_{algae}, MoS_{crustacean}, MoS_{fish})} \quad \textbf{(Eq. 7.3)}$$

Optionally, the use of in-vitro assays such as the CALUX (Chemical Activated LUciferase gene eXpression) assay could help classifying the mode of action of the sampled ERCM to identifying mixture toxicity driving chemicals or groups of chemicals within the ERCM. Alternatively, the combination of non-targeted chemical analysis and multivariate statistics is a promising approach for virtual EDA and may help identifying mixture toxicity driving substances or predicting toxic samples based on compositional similarity [165].

# 8

# General discussion and future recommendations

# 8 General discussion and future recommendations

## 8.1 Introduction

In Europe, several regulations with the aim of reducing chemical pressure on the environment are in place. Most of these regulations aim to reduce the release of chemicals into the environment by restricting the use of those with the highest risks for human or environmental health. Nevertheless, due to the wide dispersive use and manifold applications of chemicals, a high amount is constantly introduced into the environment and especially aquatic systems. Here, wastewater treatment plants are used to lower chemical's concentrations and chemical analysis is the norm for water quality assessment. This is despite the fact that an evaluation on a per-chemical basis covers only a minor fraction of known target substances and may not represent the actual removal performance [166]. Accordingly, several studies reported a remarkable discrepancy between the toxicity observed in bioassays and the toxicity predicted based on chemical analysis even when a broad set of target chemicals (> 400) was included [167-169]. Overall, the removal efficiency in wastewater treatment plants has been found to be considerably improved through application of tertiary treatment using e.g. activated carbon or ozonation. Here, the removal performance of the two methods does not differ significantly from a biological endpoint perspective [166]. Solely the generation of potentially toxic transformation products during ozonation makes activated carbon treatment preferable. Ozonation on the other hand has additional benefits including disinfection and lower implementation and maintenance costs [166].

European chemical-related regulations (e.g. the Water Framework Directive) rely on the assessment of chemical safety of a limited amount of priority pollutants. Yet, these priority pollutants represent only a minor fraction (0.2 %) of the commercially relevant and potentially emitted chemicals [128]. Environmental risk assessment of chemicals is most often still based on predicted environmental concentration (PEC) or measured environmental concentration (MEC) and effect assessment on a substance-by-substance basis. Given the fact that for many existing substances data are lacking, there is a relatively high chance of a substance's risk being unknown. Recently, this shortcoming of the European legislations has been identified and potential solutions are being discussed [63, 128].

This thesis aimed to partly address the identified shortcomings from two different perspectives. The first part targeted the problem on an individual substance basis in a retrospective approach by providing missing marine ecotoxicity data for a number of selected compounds previously detected in the Belgian Part of the North Sea (BPNS). Then, an automated calculation algorithm was developed to help prioritizing CECs for future research or refined assessment. The second part of this research targeted the identified shortcomings from a mixture-based perspective. Here, a novel methodology for an effect-based monitoring approach using passive samplers was developed. This methodology combined both retrospective effects assessment for environmentally realistic chemical mixtures (ERCMs) and Margin of Safety (MoS) assessments for the BPNS.

## 8.2 Single substance testing of chemicals of emerging concern

### *8.2.1 Findings*

**Key finding 1:** *P. tricornutum* showed low sensitivity when exposed to 1 personal care product (PCP, 72h-$EC_{50}$ > 53 mg $L^{-1}$), 7 pesticides (72h-$EC_{50}$ = 9.7 to > 160 mg $L^{-1}$) and 12 pharmaceuticals (72h-$EC_{50}$ = 6.6 to > 323 mg $L^{-1}$) individually.

**Key finding 2:** *N. spinipes* showed low sensitivity when exposed to 1 PCP (96h-$LC_{50}$ > 37 mg $L^{-1}$), 5 pesticides (96h-$LC_{50}$ = 12 to > 120 mg $L^{-1}$) and 12 pharmaceuticals (96h-$LC_{50}$ = 4.8 to > 151 mg $L^{-1}$) but high sensitivity when exposed to 4 neonicotinoid insecticides (96h-$EC_{50}$ = 0.0069 to > 0.12 mg $L^{-1}$).

**Key finding 3:** For neonicotinoid insecticides immobility was a more sensitive endpoint than lethality in *N. spinipes*.

**Key finding 4:** Exceedance of predicted no-effect concentrations (PNECs) in the studied harbors and low MoS for the coastal locations suggest the presence of environmental risks in the BPNS due to neonicotinoid insecticides.

The generated toxicity data for *N. spinipes* led to a refinement of the saltwater annual average-Environmental Quality Standard (AA-EQS) for clothianidin (CLO), and contributed to a reduction of the uncertainty in the derivation of the saltwater AA-EQS for thiamethoxam (TMX) by reducing the AF. In addition, data generated for both *P. tricornutum* and *N. spinipes* allowed the definition of PNECs for 5 additional substances when added to the input ecotoxicity data for the automated calculation algorithm (chapter 5). These results demonstrate the general need for ecotoxicity data and show that especially data for marine species can considerably reduce uncertainty when deriving saltwater-specific threshold values. In addition,

### *8.2.2 Future recommendations*

Under e.g. REACH, registration and marketing of chemicals with high tonnage (> 1 ton per year) have strict data requirements. Even though lots of ecotoxicity data has been generated for a wide range of chemicals, not all is publicly available or remains hidden or difficult to access in EU repositories. Various initiatives have identified this lack of data availability or accessibility and created large online databases (e.g. the US EPA ECOTOXicology Knowledgebase or EnviroTox). In order to support these initiatives, public availability and accessibility to (ecotoxicity) data used in chemical registration procedures should be made mandatory [170].

## 8.3 Development of an automated approach for screening-level risk assessment of chemicals of emerging concern

### *8.3.1 Findings*

**Key finding 1:** No obvious spatio-temporal differences of risk quotient (RQ) distributions have been observed.

**Key finding 2:** Comparable distributions for grab water sample and speedisk passive sampler-based RQs for pesticides and pharmaceuticals were found, but for phenols, phthalates and steroids grab water sample-based RQs are higher than speedisk-based RQs.

**Key finding 3:** All substance classes contain cases with RQ > 1 with most RQs for PCPs, pharmaceuticals and phthalates < 10, but with RQ > 100 for certain pesticides, phenols and steroids.

**Key finding 4:** For substances with higher RQs fish are often the most sensitive species group.

### *8.3.2 Future recommendations*

Our screening-level marine risk assessment suggests to prioritize in future work Bisphenol A, certain herbicides, neonicotinoid insecticides and steroids for further ecotoxicological testing and/or refined PNEC calculation. This is in good agreement with the outcome of a risk assessment for fifty pharmaceuticals and PCPs in Chinese surface waters [171]. Here, high (RQ > 10) to moderate (10 > RQ > 1) risks were identified for Ethinylestradiol (EE2), Estradiol (E2) and diethylhexyl phthalate (DEPH), and dibutyl phthalate and Bisphenol A, respectively [171]. Yet, the currently applied single substance-based risk assessment using EQS has been shown to be non-protective for organisms exposed to chemical mixtures by Carvalho et al. (2016) who exposed microalgae, daphnids, fish and frog embryos to mixtures of 14 or 19 chemicals at concentrations 100-fold or more below their individual no-observed adverse effect levels (NOAELs) [172]. Thus, in order to adequately address mixture risks, changes in current EU regulations and novel methods for mixture-based risk assessments are required.

## 8.4 Development of a novel method for passive sampler-based ecotoxicity testing of environmentally realistic chemical mixtures

### *8.4.1 Findings*

**Key finding 1:** Using speedisk extracts to spike algae growth medium in Erlenmeyer flasks resulted in ecotoxicity testing with a relative enrichment factor (REF) < 2 (as compared to environmental concentrations).

**Key finding 2:** Exposure to speedisk extracts at environmentally realistic concentrations resulted in growth stimulation of *P. tricornutum* at two sampling locations (HZ and OZ_MOW1) in one of five sampling campaigns (SCs).

**Key finding 3:** Handling and storage time of speedisk extracts needs to be reduced to a minimum in order to ensure the conservation of the original mixture composition.

**Key finding 4:** Multivariate statistics (i.e. PCA and OPLS-DA) applied to a limited set of target compound concentrations did not result in satisfactory mixture toxicity driver identification and effects may have been linked to the presence of other untargeted or unknown substances.

### *8.4.2 Future recommendations*

Our results underline the importance of a limited handling time and reduced number of manipulation steps when working with passive sampler extracts to assure maintenance of the mixture composition in such complex samples. For the identification of mixture toxicity driving substances, effect-directed analysis (EDA) has been proposed and successfully applied in various studies [23, 147, 173, 174]. EDA is a highly suitable effect-based tool to identify mixture toxicity driving chemicals but is rather limited to *in-vitro* biotest systems and therefore more applicable for an effect assessment on a lower level of biological organization (sub-organism level). If, however, the intention is to combine effect assessment on a higher level of biological organization (organism or population level), the use of multivariate statistics could lead to mixture toxicity driver identification. The condition for this approach being successful is knowledge of all potentially present substances in the mixture. Therefore, we suggest to couple multivariate statistical methods with non-target screening which allows the detection of a virtually unlimited number of compounds as an alternative non-biased chemical analytical approach.

## 8.5 A margin of safety approach for the assessment of environmentally realistic chemical mixtures in the marine environment based on combined passive sampling and ecotoxicity testing

### *8.5.1 Findings*

**Key finding 1:** Reconstitution of speedisk extracts and biotest system adaptations enabled ecotoxicity testing up to a REF of 44 as compared to environmentally realistic concentration levels.

**Key finding 2:** Samples with a REF ≥ 13 always affected the growth of *P. tricornutum* negatively while a REF ≤ 2.8 never resulted in growth inhibition.

**Key finding 3:** For 5 out of 8 samples the MoS was found to be lower than 10 suggesting ecological risks for these sampling locations.

**Key finding 4:** For samples with a REF ≥ 13 we observed cell death and thus no recovery potential for *P. tricornutum*.

### *8.5.2 Future recommendations*

The developed method (including MoS calculation) provides a methodology for a mixture-based effect assessment. While a development of the current legislation from a single substance-based to a mixture-based risk assessment would be desirable, we suggest to align the MoS approach with the current regulations until changes come into force. Therefore, we propose an adaptation of the speedisk extract-based MoS approach for the use with additional biotest systems to cover additional trophic levels and allow a mixture-based risk assessment derived from at least 3 endpoints for different representative species. These species should ideally include algae (*P. tricornutum*), crustacean (we suggest to use the 7-day larval development test with *N. spinipes*) and fish (e.g. the fish early-life stage toxicity test with *Cyprinodon variegatus*). This could form the basis for a solid risk characterization. In addition, for the sake of reducing sample consumption and test organisms, we suggest to limit the testing of speedisk extracts to a range of REF 1-10, since i) testing of this small REF range reduces the spacing factor between each REF, whilst the number of tested REF's can remain the same, ii) when considering the typical AF of 10 for lab to field extrapolation, observing no effects up to REF 10 can be considered equivalent to "no risks" and iii) testing this reduced concentration range is associated with reduced work load and costs.

## 8.6 Overall contribution of this thesis to advances in ecotoxicological research and legislation

This thesis responds to the current and future EU regulations with regards to environmental pollution. With the European Green Deal (EGD) presented in December 2019 the European Union has set the ambitious goal to achieve a non-toxic environment until 2030. Restored and properly protected marine ecosystems bring substantial health as well as social and economic benefits to coastal communities and the EU as a whole [61]. One of the key drivers of biodiversity loss is pollution. The EU has already solid legal frameworks in place to regulate chemical use and reduce pollution but greater efforts are still required [61]. Further, the Farm to Fork strategy will impose a reduction in use and risks of pesticides. This will partially be achieved by strengthening the risk assessment of pesticides. An initial monitoring campaign of the NewSTHEPS project revealed the presence of a multitude of pesticides but also PCPs and pharmaceuticals in the BPNS. A literature screening for ecotoxicity data of the detected compounds identified considerable gaps for marine ecotoxicity data. To answer this shortcoming and with the aim of improving data quality and quantity for risk assessment, ecotoxicity data for a marine diatom and a brackish copepod species was generated for a total of 23 of the detected substances. This data has been shown to and will further aid to help reducing the uncertainty in future risk assessments of these chemicals including pesticides. This has been shown in detail for the derivation of EQS and subsequent risk assessment for 4 neonicotinoid insecticides in the BPNS. This risk assessment not only identified potential risks (RQ > 1) due to the presence of individual neonicotinoids at two Belgian harbors but also revealed a relatively low MoS (RQ > 0.1) for the simultaneous presence of these four neonicotinoids for two coastal locations in the BPNS. In addition, acute toxicity testing of adult copepods with neonicotinoids revealed immobilization to be a more sensitive endpoint than mortality for *N. spinipes*. Further, short-term exposure of adults has been shown to be a comparably sensitive endpoint than long-term exposure of larvae. In summary, marine

ecotoxicity testing and risk assessment for individual chemicals of emerging concern (CECs) raised concerns with regard to the pollution status of the BPNS.

Yet, threshold derivation (e.g. EQS derivation) and risk assessment for individual substances have been shown to be time-intensive processes. To facilitate risk assessment of chemical pollution the European Commission is planning to develop a set of indicators with the aim of progressively reducing chemical pollution [61]. This should result in the definition of EQS for a broad range of chemicals to serve as benchmarks for a toxic-free environment. Nevertheless, given the multitude of chemicals present in most aquatic environments, there is a clear need for automated processes for the prioritization of CECs including both ecotoxicity and chemical monitoring data. This thesis provides an automated calculation algorithm for prioritization and screening-level risk assessment of CECs for future research. This algorithm might help to identify chemicals of concern that require risk mitigation measures in order to achieve the goal of a zero pollution environment under the EGD. The screening-level risk assessment identified Bisphenol A, certain herbicides, neonicotinoid insecticides and steroids as chemical(s) (groups) of emerging concern but also identified specific substances in other chemical groups (e.g. the phthalate DEPH) with RQ > 1. While risks for freshwater environments due to the presence of steroids or neonicotinoid insecticides are largely known, this is one of the first times that potential risks for a marine environment have been reported. In future research, the developed automated calculation algorithm should be extended to include additional ecotoxicity data from other databases (e.g. the EU Pesticides Database or the EnviroTox Database) to extend the data basis for a refined PNEC calculation and subsequent risk assessment. Further, the identified substance(s) groups with comparably high RQs should undergo an in-depth investigation and e.g. uncertainty related to e.g. high AFs be reduced by additional ecotoxicity testing or literature research. Overall, the results of the screening-level marine risk assessment highlight the need for additional investigation of the chemical status of the BPNS and should serve regulators as basis for the prioritization of future research.

A third and last major contribution of this thesis is the development of a novel passive sampler-based MoS approach for effect-based risk assessment of ERCMs. Here, an enrichment of speedisk extracts relative to environmentally realistic chemical concentrations above 13 always resulted in growth inhibition but effects were observed as of a REF >2.8 in some cases. Generally, MoS below 10 were found for 5 out of 8 sampling locations indicating potential mixture risks according to conventional risk assessment. This method provides an effective tool for an effect-based monitoring of chemical mixtures. So far, the system has only been adapted to algae growth inhibition testing but an adaptation to other small-scale biotest systems for species representing other trophic levels is highly recommended. Also focusing on a range of REF 1-10 is highly recommended. This is based on the lowest AF applied on single-species derived thresholds being 10. The observation of an effect for a sample enriched to REF ≤ 10 would thus be equivalent to a risk. In addition, we used PCA to explore the chemical data from the tested speedisk extracts to find potential mixture toxicity drivers. Research into this direction would further be beneficial in order to link observed toxicity to specific chemicals or chemical groups. In this regard, the European Food Safety Authority (EFSA) published a guidance document on harmonized methodologies for risk assessment of combined exposure to multiple chemicals [175]. This guidance document distinguishes between a whole mixture approach for mixtures with known chemical composition and component-based approaches for such mixtures with only partially known chemical composition. Certainly, the whole mixture approach rarely finds application in environmental research since chemical mixtures found in especially aquatic environments are usually

complex and of (partially) unknown composition. For component-based approaches, an important consideration is whether and how to account for potential interactions between components [175]. EFSA proposed to generally adopt the concept of concentration addition since it has been shown to be applicable to a wide range of endpoints and it provides reliable approximations of observed combination effects [176]. It is recognized that data gaps may be highly variable across different scenarios including missing exposure or hazard data, or both [175]. To fill such data gaps, methods developed for individual chemical assessments (e.g. *in-silico* models like QSARs or read-across) have also been proposed for mixtures. In this case, attention must be dominating to avoid potential over-interpretation of risks related to high uncertainty factors [175]. The outcomes of such risk assessments should always be scrutinized for interpretation bias by identifying the substances that contributed the most to identified risks [175]. Final recommendations for future work to support filling data gaps of the proposed guidance document included i) further development and implementation of publicly accessible databases and tools for exposure and hazard assessment of multiple chemicals, ii) further implementation of probabilistic exposure assessment methodologies for multiple chemicals, iii) further development of non-target chemical analysis for the characterization of chemical mixtures and iv) provision of better integration of high throughput, *in vitro* data generated from modern technologies to integrate data from alternative methods under the 3R principles (replacement, reduction and refinement). These recommendations certainly (partly) align with the recommendations given in this thesis and guide the way for future mixture toxicity-based risk assessment practices.

Overall, the here presented research provides Belgian authorities with novel insights about the BPNS that may help prioritizing future research to better understand Descriptor 8 of the WFD. Ecotoxicity testing and (screening-level) risk assessment of both individual substances and ERCMs have shown that today's concentrations of contaminants in the BPNS are at levels giving rise to pollution effects. In addition, the automated screening-level risk assessment provides useful information about potentially problematic chemical(s) groups. The underlying code should be seen as a tool under development that can continuously be improved and should serve as screening tool for future monitoring of Belgian waters. With the ambitious goal of a zero-pollution environment under the EGD, the Belgian authorities are strongly advised to move from an individual substance-based to a mixture-based risk assessment. Only effect-based approaches are suitable to monitor the actual status of (Belgian) waters and, if extended with adequate (non-target) analytical methods, may be used to identify chemicals of emerging concern.

# Annex A

Supportive information to Chapter 4

# Annex A

## A1 Materials and methods

### A1.1 Overview of NewSTHEPS target substances

**Table A1 Overview of the target chemicals of the NewSTHEPS project. PS describes Water Framework Directive priority substances.**

| Compound name (salt) | Subclass | CAS-number | Log $K_{ow}$[a] (at 25°C) | pKa[b] (at 25°C) | MSFD target substance[c] | Algae testing | Copepod testing |
|---|---|---|---|---|---|---|---|
| **Personal care products** | | | | | | | |
| Butylparaben | Preservative | 94-26-8 | 3.57 | 8.22 | NO | NO | NO |
| Chloroxylenol | Disinfectant | 88-04-0 | 3.27 | 9.76 | NO | NO | NO |
| DEET | Insect repellent | 134-62-3 | 2.02 | -1.37 | NO | YES | YES |
| Ethylparaben | Preservative | 120-47-8 | 2.47 | 8.31 | NO | NO | NO |
| Methylparaben | Preservative | 99-76-3 | 1.96 | 8.31 | NO | NO | NO |
| Propylparaben | Preservative | 94-13-3 | 3.04 | 8.23 | NO | NO | NO |
| Oxybenzone | UV filtering compound | 131-57-7 | 3.79 | 7.56 | NO | NO | NO |
| Piperonylbutoxide | Insect repellent | 51-03-6 | 4.75 | - | NO | NO | NO |
| Triclosan | Disinfectant | 3380-34-5 | 4.76 | 7.8 | NO | NO | NO |
| **Pesticides** | | | | | | | |
| Acetamiprid | Neonicotinoid insecticide | 135410-20-7 | 0.80 | -0.44 | YES | NO | NO |
| Aclonifen | Herbicide | 74070-46-5 | 3.28 | -3.15 | YES (PS) | NO | NO |
| Alachlor | Herbicide | 15972-60-8 | 3.52 | 1.2 | YES (PS) | YES | YES |
| Atrazine | Herbicide | 1912-24-9 | 2.61 | 2.27 | YES (PS) | NO | NO |
| Chlorfenvinphos | Organophosphate insecticide | 470-90-6 | 3.81 | - | YES (PS) | NO | NO |
| Chloridazon | Herbicide | 1698-60-8 | 1.14 | 0.71 | YES | YES | YES |
| Clothianidin | Neonicotinoid insecticide | 210880-92-5 | 0.70 | -0.20; 2.76 | YES | NO | YES |
| 2,4-D | Herbicide | 94-75-7 | 2.81 | 2.98 | YES | NO | NO |
| Dichlorvos | Organophosphate insecticide | 62-73-7 | 1.43 | - | YES | NO | NO |
| Dimethoate | Organophosphate insecticide | 60-51-5 | 0.78 | -0.98; 14.4 | YES | NO | NO |
| Dinoseb | Herbicide | 88-85-7 | 3.56 | 4.08 | NO | NO | NO |
| Diuron | Herbicide | 330-54-1 | 2.68 | -1.09; 13.55 | YES (PS) | NO | NO |
| Flufenacet | Herbicide | 142459-58-3 | 3.20 | 0.31 | NO | YES | YES |
| Imidacloprid | Neonicotinoid insecticide | 138261-41-3 | 0.57 | 4.80; 7.16 | YES | YES | YES |
| Irgarol | Fungicide | 28159-98-0 | 3.26 | 4.13 | YES (PS) | NO | NO |
| Isoproturon | Herbicide | 34123-59-6 | 2.89 | 0.85; 15.06 | YES (PS) | NO | NO |
| Linuron | Herbicide | 330-55-2 | 3.20 | -1.04; 12.13 | YES | NO | NO |

| Compound name (salt) | Subclass | CAS-number | Log $K_{ow}$[a] (at 25°C) | pKa[b] (at 25°C) | MSFD target substance[c] | Algae testing | Copepod testing |
|---|---|---|---|---|---|---|---|
| Mecoprop | Herbicide | 7085-19-0 | 3.13 | 3.19 | YES | YES | YES |
| Methiocarb | Carbamate insecticide | 2032-65-7 | 2.92 | -1.52; 12.16 | NO | NO | NO |
| Metolachlor | Herbicide | 51218-45-2 | 3.13 | 1.45 | YES | NO | NO |
| Pentachlorophenol | Organochlorine insecticide | 87-86-5 | 5.12 | 4.68 | YES (PS) | NO | NO |
| Pirimicarb | Carbamate insecticide | 23103-98-2 | 1.70 | 5.00 | YES | YES | YES |
| Quinoxyfen | Fungicide | 124495-18-7 | 4.66 | 2.87 | YES | NO | NO |
| Simazine | Herbicide | 122-34-9 | 2.18 | 2.71 | YES (PS) | NO | NO |
| Terbuthylazine | Herbicide | 5915-41-3 | 3.40 | 2.69 | YES | NO | NO |
| Terbutryn | Herbicide | 886-50-0 | 3.74 | 4.03 | YES (PS) | NO | NO |
| Thiacloprid | Neonicotinoid insecticide | 111988-49-9 | 1.26 | 0.01 | YES | NO | NO |
| Thiamethoxam | Neonicotinoid insecticide | 153719-23-4 | -0.13 | 0.99 | YES | YES | YES |
| **Pharmaceuticals** | | | | | | | |
| Acetylsalicylic acid | Analgesic | 50-78-2 | 1.19 | 3.48 | NO | NO | NO |
| Acyclovir | Antiviral | 59277-89-3 | -1.56 | 2.55; 9.35 | NO | NO | NO |
| Alprazolam | Tranquilizer | 28981-97-7 | 2.12 | 2.37 | NO | NO | NO |
| Amantadine (HCl) | Antiviral | 768-94-5 | 2.44 | 10.76 | NO | YES | YES |
| Amitriptyline (HCl) | Antidepressant | 50-48-6 | 4.92 | 9.18 | NO | NO | NO |
| Atenolol | β-blocker | 29122-68-7 | 0.16 | 9.43; 13.88 | NO | YES | YES |
| Azithromycin ($2H_2O$) | Macrolide antibiotic | 83905-01-5 | 4.02 | 8.59; 13.28 | YES | NO | NO |
| Bezafibrate ($H_2O$) | Lipid regulator | 41859-67-0 | 2.50 | -2.06; 3.29 | NO | YES | YES |
| Bisoprolol | β-blocker | 66722-44-9 | 1.87 | 9.42; 13.86 | NO | NO | NO |
| Carbamazepine | Anti-epilepticum | 298-46-4 | 2.45 | -0.49; 13.94 | YES | YES | YES |
| Carprofen | Non-steroidal anti-inflammatory drug | 53716-49-7 | 3.80 | -5.73; 4.84 | NO | NO | NO |
| Chloramphenicol | Broad-spectrum antibiotic | 56-75-7 | 1.14 | -1.73; 11.03 | NO | NO | NO |
| Ciprofloxacin (HCl) | Quinolone antibiotic | 85721-33-1 | 0.28 | 8.68; 6.43 | NO | NO | NO |
| Clarithromycin | Macrolide antibiotic | 81103-11-9 | 3.16 | 8.16; 13.08 | YES | NO | NO |
| Clofibric acid | Lipid regulator | 882-09-7 | 2.43 | 3.18 | NO | NO | NO |
| Diazepam | Tranquilizer | 439-14-5 | 2.82 | 3.4 | NO | NO | NO |
| Diclofenac (Na salt) | Non-steroidal anti-inflammatory drug | 15307-86-5 | 4.51 | -2.26; 4.18 | YES | YES | YES |
| Efavirenz | Antiviral | 154598-52-4 | 4.60 | -1.89; 10.24 | NO | NO | NO |
| Enrofloxacin | Quinolone antibiotic | 93106-60-6 | 2.31 | 6.43; 7.76 | NO | NO | NO |
| Flumequine | Quinolone antibiotic | 42835-25-6 | 1.60 | -1.98; 5.7 | NO | YES | YES |
| Fluoxetine (HCl) | Antidepressant | 54910-89-3 | 4.05 | 10.05 | NO | NO | NO |
| Gatifloxacin | Quinolone antibiotic | 112811-59-3 | 2.60 | 6.43; 8.72 | NO | NO | NO |
| Ibuprofen | Non-steroidal anti-inflammatory drug | 15687-27-1 | 3.97 | 4.41 | NO | NO | NO |
| Ifosfamide | Alkylating agent | 3778-73-2 | 0.86 | 1.44 | NO | NO | NO |
| Indomethacin | Non-steroidal anti-inflammatory drug | 53-86-1 | 4.27 | 3.96 | NO | NO | NO |

| Compound name (salt) | Subclass | CAS-number | Log $K_{ow}$[a] (at 25°C) | pKa[b] (at 25°C) | MSFD target substance[c] | Algae testing | Copepod testing |
|---|---|---|---|---|---|---|---|
| Ketoprofen | Non-steroidal anti-inflammatory drug | 22071-15-4 | 3.12 | 4.23 | NO | NO | NO |
| Lamivudine | Antiviral | 134678-17-4 | -1.40 | 4.18; 13.83 | NO | NO | NO |
| Levofloxacin | Quinolone antibiotic | 100986-85-4 | 2.10 | 5.19; 7.37 | NO | NO | NO |
| Metoprolol ($C_4H_6O_6$) | β-blocker | 51384-51-1 | 1.88 | 4.52; 13.89 | NO | YES | YES |
| Metronidazole | Broad-spectrum antibiotic | 443-48-1 | -0.02 | 2.58; 14.44 | NO | NO | NO |
| Moxifloxacin (HCl) | Quinolone antibiotic | 151096-09-2 | 2.90 | 6.43; 10.63 | NO | YES | YES |
| Nalidixic acid | Quinolone antibiotic | 389-08-2 | 1.59 | 3.45; 6.12 | NO | NO | NO |
| Naproxen | Non-steroidal anti-inflammatory drug | 22204-53-1 | 3.18 | 4.84 | NO | NO | NO |
| Nevirapine | Antiviral | 129618-40-2 | 2.50 | 4.25; 12.05 | NO | NO | NO |
| Oseltamivir acid | Antiviral | 187227-45-8 | 0.74 | 4.13; 9.26 | NO | NO | NO |
| Oseltamivir ethylester | Antiviral | 196618-13-0 | 1.71 | 8.81; 14.68 | NO | NO | NO |
| Oxytetracycline (HCl) | Tetracycline antibiotic | 79-57-2 | -0.90 | 4.50; 10.8 | NO | YES | YES |
| Paracetamol | Analgesic | 103-90-2 | 0.46 | 1.72; 9.86 | NO | YES | YES |
| Paroxetine ($HCl.5H_2O$) | Antidepressant | 61869-08-7 | 3.60 | 9.68 | NO | NO | NO |
| Propranolol (HCl) | β-blocker | 525-66-6 | 3.48 | 9.50; 13.84 | NO | NO | NO |
| Rimantadine (HCl) | Antiviral | 13392-28-4 | 3.60 | 11.17 | NO | NO | NO |
| Salbutamol | Adrenergic | 18559-94-9 | 1.40 | 9.62; 9.99 | NO | NO | NO |
| Salicylic acid | Analgesic | 69-72-7 | 2.26 | 3.01 | NO | NO | NO |
| Sarafloxacin ($HCl.3H_2O$) | Quinolone antibiotic | 98105-99-8 | 1.07 | 6.17; 8.68 | NO | NO | NO |
| Sotalol (HCl) | β-blocker | 3930-20-9 | 0.24 | 8.28; 9.31 | NO | NO | NO |
| Sulfadoxine | Sulfonamide antibiotic | 2447-57-6 | 0.70 | 2.18; 6.16 | NO | NO | NO |
| Sulfamethazine | Sulfonamide antibiotic | 57-68-1 | 0.89 | 1.69; 7.89 | NO | NO | NO |
| Sulfamethoxazole | Sulfonamide antibiotic | 723-46-6 | 0.89 | 1.39; 5.81 | NO | YES | YES |
| Tetracycline (HCl) | Tetracycline antibiotic | 60-54-8 | -1.30 | 4.50; 11.02 | NO | NO | NO |
| Trimethoprim | Sulfonamide antibiotic | 738-70-5 | 0.91 | 7.04 | NO | NO | NO |
| Venlafaxine (HCl) | Antidepressant | 93413-69-5 | 3.20 | 9.26; 14.84 | NO | YES | YES |
| Zidovudine | Antiviral | 30516-87-1 | 0.05 | - | NO | YES | YES |
| **Steroidal EDCs** | | | | | | | |
| Methandriol | Androgens | 521-10-8 | 4.32 | 15.01 | NO | NO | NO |
| 17α-trenbolone | Androgens | 80657-17-6 | 2.31 | 14.73 | NO | NO | NO |
| 17β-trenbolone | Androgens | 10161-33-8 | 3.16 | 14.73 | NO | NO | NO |
| 11β-hydroxyandrosterone | Androgens | 57-61-4 | 1.97 | 14.62 | NO | NO | NO |
| Testosterone 17β-cypionate | Androgens | 58-20-8 | 6.60 | - | NO | NO | NO |
| 17β-dihydroandrosterone | Androgens | 1852-53-5 | 4.39 | 15.07 | NO | NO | NO |
| Androsterone | Androgens | 53-41-8 | 3.93 | 15.15 | NO | NO | NO |
| 19-nortestosterone | Androgens | 434-22-0 | 2.89 | 15.06 | NO | NO | NO |

| Compound name (salt) | Subclass | CAS-number | Log $K_{ow}$[a] (at 25°C) | pKa[b] (at 25°C) | MSFD target substance[c] | Algae testing | Copepod testing |
|---|---|---|---|---|---|---|---|
| 1,4-Androstadienedione | Androgens | 897-06-3 | 2.62 | - | NO | NO | NO |
| 11-ketoetiocholanolone | Androgens | 739-27-5 | 1.89 | 15.07 | NO | NO | NO |
| Androstenedione | Androgens | 63-05-8 | 2.71 | - | NO | NO | NO |
| Mestanolone | Androgens | 521-11-9 | 4.31 | 15.15 | NO | NO | NO |
| 17α-testosterone | Androgens | 481-30-1 | 3.17 | 15.06 | NO | NO | NO |
| 17β-testosterone | Androgens | 58-22-0 | 3.17 | 15.06 | NO | NO | NO |
| 5α-dihydrotestosterone | Androgens | 521-18-6 | 3.93 | 15.08 | NO | NO | NO |
| 19-Norethindron | Androgens | 68-22-4 | 2.86 | 13.09 | NO | NO | NO |
| Methylboldenone | Androgens | 72-63-9 | 3.47 | 15.12 | NO | NO | NO |
| 11-ketotestosterone | Androgens | 564-35-2 | 1.30 | 14.79 | NO | NO | NO |
| Formestane | Androgens | 566-48-3 | 1.79 | 9.31 | NO | NO | NO |
| Norethandrolone | Androgens | 52-78-8 | 3.77 | 15.13 | NO | NO | NO |
| Methyltestosterone | Androgens | 58-18-4 | 3.55 | 15.13 | NO | NO | NO |
| Trenbolone acetate | Androgens | 10161-34-9 | 4.01 | - | NO | NO | NO |
| Ethynyl testosterone | Androgens | 434-03-7 | 3.13 | 13.10 | NO | NO | NO |
| Stanozolol | Androgens | 10418-03-8 | 5.41 | 15.15 | NO | NO | NO |
| Testosterone acetate | Androgens | 434-03-7 | 3.13 | 13.10 | NO | NO | NO |
| Fluoxymesterone | Androgens | 76-43-7 | 2.26 | 13.40 | NO | NO | NO |
| Testosterone propionate | Androgens | 911657-75-5 | 4.39 | - | NO | NO | NO |
| Chlorotestosteron acetate | Androgens | 855-19-6 | 4.60 | - | NO | NO | NO |
| Testosterone benzoate | Androgens | 42723-70-6 | 5.99 | - | NO | NO | NO |
| Testosterone phenylpropionate | Androgens | 1255-49-8 | 6.28 | - | NO | NO | NO |
| 19-nortestosterone-17-decanoate | Androgens | 360-70-3 | 7.93 | - | NO | NO | NO |
| 17α-estradiol | Oestrogens | 57-91-0 | 4.14 | 10.27 | NO | NO | NO |
| 17β-estradiol | Oestrogens | 50-28-2 | 4.14 | 10.27 | YES | NO | NO |
| Estradiol-17-acetate | Oestrogens | 1743-60-8 | 5.11 | 10.26 | NO | NO | NO |
| Dienoestrol | Oestrogens | 84-17-3 | 4.92 | 9.21 ± | NO | NO | NO |
| Equilin | Oestrogens | 474-86-2 | 2.27 | 10.11 | NO | NO | NO |
| Diethylstilbestrol | Oestrogens | 56-53-1 | 5.33 | 10.18 | NO | NO | NO |
| Estrone | Oestrogens | 53-16-7 | 3.62 | 10.25 | YES | NO | NO |
| 17α-ethinylestradiol | Oestrogens | 57-63-6 | 4.10 | 10.24 | YES | NO | NO |
| α-zearalenol | Oestrogens | 36455-72-8 | 4.16 | 7.61 | NO | NO | NO |
| β-zearalenol | Oestrogens | 71030-11-0 | 4.16 | 7.61 | NO | NO | NO |
| α-zeranol | Oestrogens | 26538-44-3 | 3.08 | 8.08 | NO | NO | NO |
| β-zeranol | Oestrogens | 42422-68-4 | 3.08 | 8.08 | NO | NO | NO |
| Gestodene | Oestrogens | 60282-87-3 | 2.02 | 12.16 | NO | NO | NO |

| Compound name (salt) | Subclass | CAS-number | Log $K_{ow}$[a] (at 25°C) | pKa[b] (at 25°C) | MSFD target substance[c] | Algae testing | Copepod testing |
|---|---|---|---|---|---|---|---|
| Estradiol-benzoate | Oestrogens | 50-50-0 | 5.09 | 15.06 | NO | NO | NO |
| 5α-Pregnan-3α,20β-diol | Progestins | 21152-50-1 | 5.44 | -3.50 | NO | NO | NO |
| Norgestrel | Progestins | 797-63-7 | 3.36 | 13.09 | NO | NO | NO |
| Dihydroprogesterone | Progestins | 165036-75-9 | 0.56 | 14.54 | NO | NO | NO |
| Progesterone | Progestins | 57-83-0 | 3.82 | - | NO | NO | NO |
| Methylprogesterone | Progestins | 896438-14-5 | 5.20 | 3.09 | NO | NO | NO |
| 17α-hydroxyprogesterone | Progestins | 68-96-2 | 3.04 | 13.03 | NO | NO | NO |
| Megestrol | Progestins | 3562-63-8 | 3.22 | 13.00 | NO | NO | NO |
| Medroxyprogesterone | Progestins | 520-85-4 | 3.57 | 13.03 | NO | NO | NO |
| 17α-acetoxyprogesterone | Progestins | 302-23-8 | 3.63 | - | NO | NO | NO |
| Megestrol acetate | Progestins | 595-33-5 | 3.74 | - | NO | NO | NO |
| Medroxyprogesterone acetate | Progestins | 71-58-9 | 4.17 | - | NO | NO | NO |
| Flugestone acetate | Progestins | 2529-45-5 | 2.81 | 13.09 | NO | NO | NO |
| Caproxyprogesterone | Progestins | 630-56-8 | 5.67 | - | NO | NO | NO |
| Prednisone | Corticosteroids | 53-03-2 | 1.56 | 12.36 | NO | NO | NO |
| Corticosterone | Corticosteroids | 50-22-6 | 1.95 | 12.98 | NO | NO | NO |
| Cortisone | Corticosteroids | 53-06-5 | 1.43 | 12.37 | NO | NO | NO |
| Prednisolone | Corticosteroids | 50-24-8 | 1.63 | 12.47 | NO | NO | NO |
| Cortisol | Corticosteroids | 50-23-7 | 1.76 | 12.47 | NO | NO | NO |
| Tetrahydrocortisone | Corticosteroids | 53-05-4 | 2.10 | 12.38 | NO | NO | NO |
| Corticosterone acetate | Corticosteroids | 1173-26-8 | 2.30 | 14.48 | NO | NO | NO |
| Dexamethasone | Corticosteroids | 50-02-2 | 2.03 | 12.13 | NO | NO | NO |
| Prednisolone acetate | Corticosteroids | 52-21-1 | 2.25 | 12.41 | NO | NO | NO |
| Cortisone acetate | Corticosteroids | 50-04-4 | 2.05 | 12.32 | NO | NO | NO |
| Hydrocortisone 21-acetate | Corticosteroids | 50-03-3 | 2.38 | 12.42 | NO | NO | NO |
| **(Alkyl)phenols** | | | | | | | |
| 2-methyl phenol | | 106-44-5 | 2.06 | 10.21 | YES | NO | NO |
| 4-ethylphenol | | 123-07-9 | 2.57 | 10.26 | NO | NO | NO |
| 4-isopropyl phenol | | 99-89-8 | 2.98 | 10.19 | NO | NO | NO |
| 4-chloro-3-methylphenol | | 59-50-7 | 2.89 | 9.63 | NO | NO | NO |
| 2,5-dichlorophenol | | 583-78-8 | 3.02 | 7.53 | NO | NO | NO |
| 3,4,6-trichlorophenol | | 88-06-2 | 3.76 | 6.59 | YES | NO | NO |
| Bisphenol A | | 65-85-0 | 1.55 | 4.20 | NO | NO | NO |
| **Phthalates** | | | | | | | |
| Dimethyl phthalate | Di-phthalates | 131-11-3 | 1.69 | - | YES | NO | NO |
| Diethyl phthalate | Di-phthalates | 84-66-2 | 2.71 | - | YES | NO | NO |

| Compound name (salt) | Subclass | CAS-number | Log $K_{ow}$[a] (at 25°C) | pKa[b] (at 25°C) | MSFD target substance[c] | Algae testing | Copepod testing |
|---|---|---|---|---|---|---|---|
| Dibutyl phthalate | Di-phthalates | 84-74-2 | 4.75 | - | YES | NO | NO |
| Diamyl phthalate | Di-phthalates | 131-18-0 | 5.77 | - | YES | NO | NO |
| Benzyl butyl phthalate | Di-phthalates | 85-68-7 | 4.91 | - | YES | NO | NO |
| Dicyclohexyl phthalate | Di-phthalates | 84-61-7 | 5.63 | - | NO | NO | NO |
| Dihexyl phthalate | Di-phthalates | 84-75-3 | 6.79 | - | YES | NO | NO |
| Dibenzyl phthalate | Di-phthalates | 523-31-9 | 5.06 | - | NO | NO | NO |
| Diethylheyxl phthalate | Di-phthalates | 117-81-7 | 8.54 | - | YES | NO | NO |
| Dinonyl phthalate | Di-phthalates | 84-76-4 | 9.84 | - | NO | NO | NO |
| Diisodecyl phthalate | Di-phthalates | 26761-40-0 | - | - | YES | NO | NO |
| Monomethyl phthalate | Mono-phthalates | 4376-18-5 | 1.13 | 3.32 | NO | NO | NO |
| Monoethyl phthalate | Mono-phthalates | 2306-33-4 | 1.63 | 3.32 | NO | NO | NO |
| Monotbutyl phthalate | Mono-phthalates | 131-70-4 | 2.65 | 3.38 | NO | NO | NO |
| Mono-n-pentyl phthalate | Mono-phthalates | 24539-56-8 | 3.16 | 3.38 | NO | NO | NO |
| Monocyclohexyl pht. | Mono-phthalates | 7517-36-4 | 3.10 | 3.29 | NO | NO | NO |
| Monohexyl phthalate | Mono-phthalates | 24539-57-9 | 3.67 | 3.39 | NO | NO | NO |
| Monobenzyl phthalate | Mono-phthalates | 2528-16-7 | 2.81 | 3.37 | NO | NO | NO |
| Monoethylhexyl phthalate | Mono-phthalates | 4376-20-9 | 4.45 | 3.37 | NO | NO | NO |
| Mono-isonyl phthalate | Mono-phthalates | 68515-53-7 | - | - | NO | NO | NO |

[a] Data retrieved from PubChem Compound database.

[b] Data retrieved from SciFinder.

[c] Marine chemical contaminants – support to harmonized MSFD reporting. Substances considered for MSFD descriptor 8 [177]

### A1.2 Derivation or Environmental Quality Standards

The ECOTOX database integrates three previously independent databases into one and includes toxicity data derived predominantly from the peer-reviewed literature (US EPA 2019). The OPP database compiles data from actual studies reviewed by EPA in conjunction with pesticide registration or re-registration. It is primarily composed of unpublished data that has been reviewed by the US EPA and thus includes additional data to what is available in the ECOTOX database. Data in the OPP database is derived from three principal sources: i) Toxicological studies conducted and submitted by pesticide companies in support of their product, ii) Studies conducted by the US EPA, Department of Agriculture, and Fish and Wildlife Service research laboratories and iii) Published studies obtained and evaluated by OPP biologists (US EPA 2018). The applied search criteria for both databases were the following:

Search criterion for the OPP database was simply the substance's CAS number. For the ECOTOX database following search criteria were applied:

1. CAS number of the substance of interest
2. Effect groups: growth, mortality, population or reproduction
3. Endpoints: ACxx, LC/LDxx, EC/EDxx, IC/IDxx, NOEC or NOEL
4. Species: All
5. Test conditions: All
6. Exposure media: water

All other search criteria were left as default. After retrieval of data from both databases, data sets were merged into a single table and final data was refined as follows:

1. All data for pesticide formulations was removed to remain with data for the pure AI for all substances.
2. All data with endpoints other than $LC_{10}$, $LC_{50}$, $EC_{10}$, $EC_{50}$, $IC_{10}$, $IC_{50}$, NOEC and NOEL was discarded.
3. All data for terrestrial organisms (e.g. bees) was removed from the data set since these organisms were not of importance for the aquatic environment.

The database exports including all data considered for the PNEC derivation for the four neonicotinoid insecticides can be consulted on the Marine Data Archive via http://mda.vliz.be/directlink.php?fid=VLIZ_00000535_5dd3eba4c6c5b.

Data from organisms belonging to a different crustacean order (amphipoda, cladocera, decapoda, isopoda, mysida and harpacticoida) was treated as data from separate taxa because life form and feeding strategy varied considerably between the different species from these orders. For algae and fish, no differentiation was made between orders and data for the most sensitive species was used when data for more than one species of the groups was available. All other taxa, i.e. insects and molluscs, were treated as separate taxa.

The TGD for deriving EQS gives detailed instructions on combining freshwater and marine species data for PNEC derivation (European Commission 2011). In short, all toxicity data was first logarithmically transformed. Then, we tested for each neonicotinoid insecticide ifthe log-transformed freshwater and saltwater toxicity dataset exhibited equal variance using an F-test ($\alpha = 0.05$). Thisshowed equal variance for all four neonicotinoids ($p \geq 0.3$). Next, two-tailed t-tests ($\alpha = 0.05$) were performed to test for differences between the freshwater and marine datasets. This resulted in no significant difference in sensitivity ($p \geq 0.42$) and the freshwater and marine datasets were combined for PNEC derivation for all four neonicotinoids.

## A2 Results

### A2.1 Growth inhibition testing with *P. tricornutum*

Figures A1 Figure A2 show the algae growth inhibition concentration-response curves for the CECs.

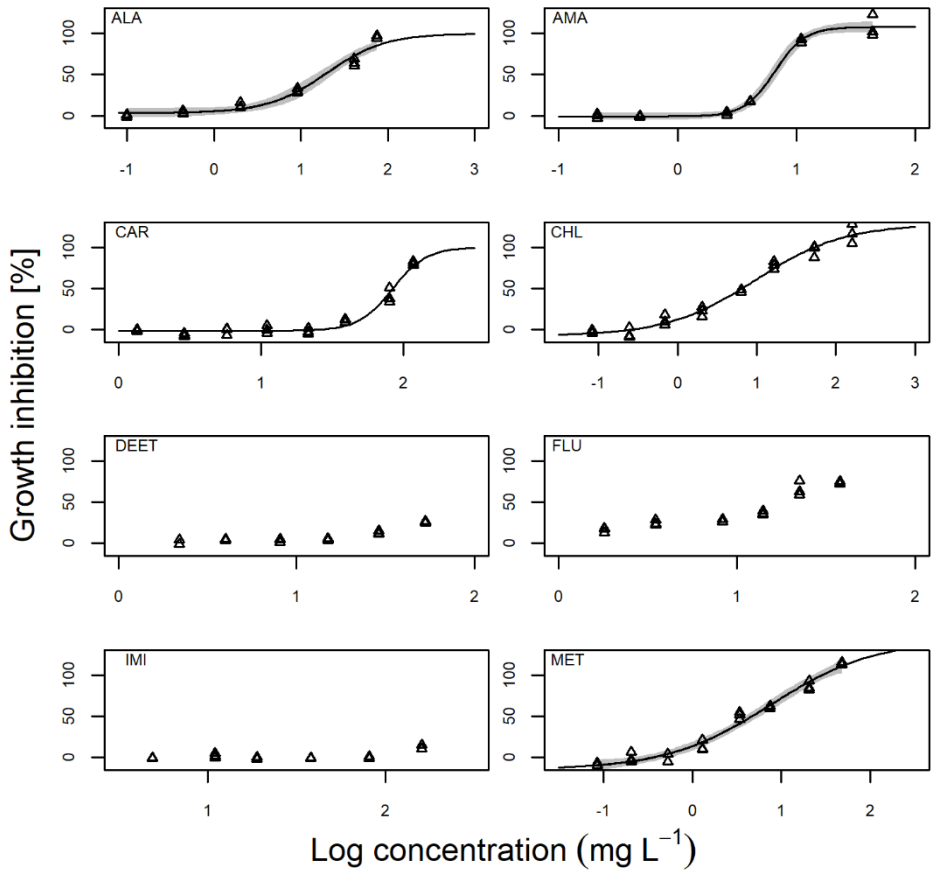


**Figure A1 Concentration-response plots for alachlor (ALA), amantadine (AMA), carbamazepine (CAR), Chloridazon (CHL), DEET (DEET), flufenacet (FLU), imidacloprid (IMI) and metoprolol (MET). Shown are the individual data points per concentration and the fitted log-logistic model (if applicable).**

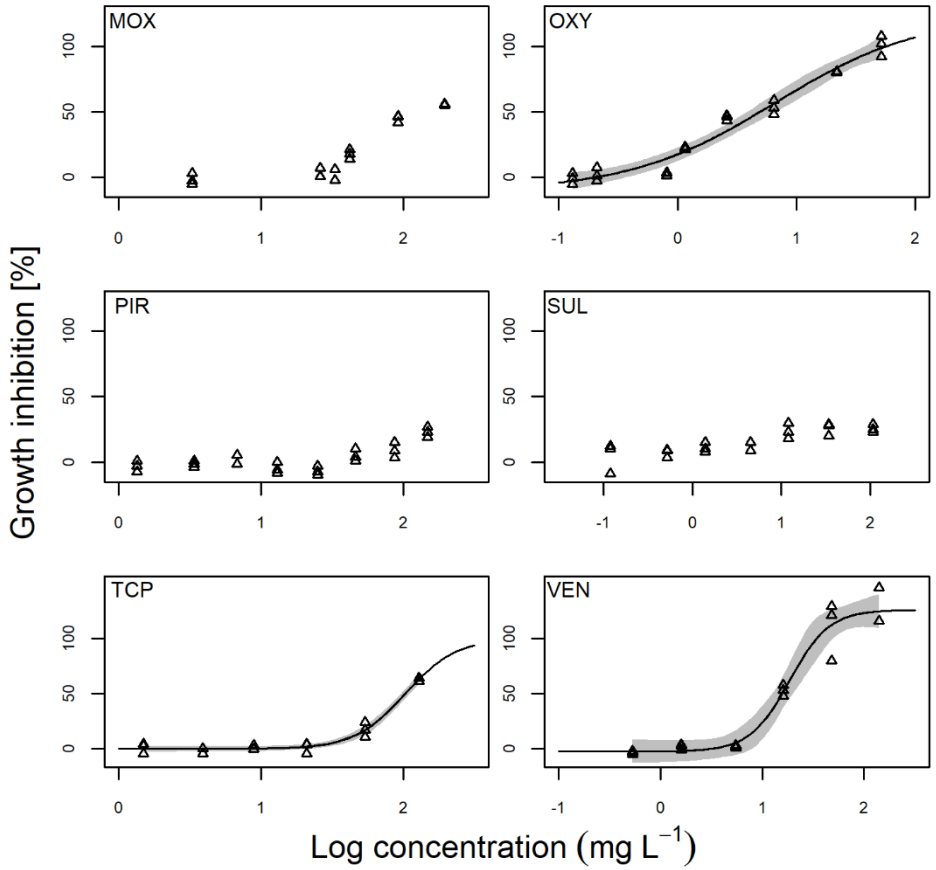


**Figure A2 Concentration-response plots for moxifloxacin (MOX), oxytetracycline (OXY), pirimicarb (PIR), sulfamethoxazole (SUL), thiacloprid (TCP) and venlafaxine (VEN). Shown are the individual data points per concentration and the fitted log-logistic model (if applicable).**

**Table A2 Overview of the test setup for the algae growth inhibition testing with *Phaeodactylum tricornutum* including the pH measurements. Shown are the lowest ($C_{min.}$) and highest ($C_{max.}$) test concentrations (measured average of triplicates unless marked as "n" = nominal), the number of treatments and the pH measurements in the lowest (pH $C_{min.}$) and highest (pH $C_{max.}$) treatments (test start – test end). All concentrations are indicated in mg $L^{-1}$. NA indicates not applicable.**

| Substance | $C_{min.}$ | $C_{max.}$ | Treatments | pH $C_{min.}$ | pH $C_{max.}$ |
|---|---|---|---|---|---|
| Alachlor | 0.099 | 75 | 6 | 7.75 – 8.62 | 7.90 – 8.20 |
| Amantadine | 0.21 | 44 | 6 | 7.76 – 8.63 | 8.20 – 8.26 |
| Bezafibrate | 1$^{n}$ | 355$^{n}$ | 6 | 7.98 – 8.26 | 7.88 – 8.25 |
| Carbamazepine | 1.4 | 117 | 8 | 7.82 – 8.29 | 7.81 – 8.13 |
| Chloridazon | 0.084 | 162 | 8 | 7.81 – 8.48 | 7.81 – 8.13 |
| DEET | 2.2 | 53 | 6 | 7.73 – 8.56 | 7.87 – 8.36 |
| Diclofenac | 1.0$^{n}$ | 355$^{n}$ | 6 | 7.98 – 8.26 | 7.88 – 8.25 |
| Flufenacet | 1.8 | 38 | 6 | 7.82 – 8.27 | 7.84 – 8.13 |
| Flumequine | 0.2$^{n}$ | 100$^{n}$ | 8 | 7.72 – 8.39 | 7.73 – 8.37 |

| Substance | $C_{min.}$ | $C_{max.}$ | Treatments | pH $C_{min.}$ | pH $C_{max.}$ |
|---|---|---|---|---|---|
| Imidacloprid | 4.9 | 160 | 6 | 7.73 – 8.64 | 7.89 – 8.45 |
| Mecoprop | 0.001[n] | 100[n] | 6 | 7.91 – 8.31 | 7.97 – 8.24 |
| Metoprolol | 0.085 | 48 | 8 | 7.63 – 8.43 | 7.69 – 8.36 |
| Moxifloxacin | 3.3 | 195 | 6 | 8.09 – 8.26 | 8.10 – 8.35 |
| Oxytetracycline | 0.13 | 52 | 8 | 8.10 – 8.26 | 8.11 – 8.29 |
| Pirimicarb | 1.35 | 148 | 8 | 7.85 – 8.31 | 7.84 – 8.21 |
| Sulfamethoxazole | 0.12 | 323 | 8 | 7.82 – 8.22 | 8.09 – 8.08 |
| Thiacloprid | 1.5 | 129 | 6 | 7.81 – 8.41 | 7.80 – 8.14 |
| Venlafaxine | 0.53 | 141 | 6 | 7.80 – 8.45 | 7.86 – 8.11 |
| Zidovudine | 1.0[n] | 100[n] | 6 | 7.80 – 8.47 | 7.84 – 8.41 |

### A2.2 Acute lethality testing with *N. spinipes*

**Table A3 Overview of the test setup for the acute lethality testing with *Nitocra spinipes* including the pH measurements. Shown are the lowest ($C_{min.}$) and highest ($C_{max.}$) nominal test concentrations, the number of treatments, the concentration factor (C-factor) applied between different treatments and the pH measurements in the lowest (pH $C_{min.}$) and highest (pH $C_{max.}$) treatments (test start – test end). All concentrations are indicated in mg $L^{-1}$. NA indicates not applicable.**

| Substance | $C_{min.}$ | $C_{max.}$ | Treatments | C-factor | pH $C_{min.}$ | pH $C_{max.}$ |
|---|---|---|---|---|---|---|
| Alachlor | 1 | 100 | 6 | 2.5 | 7.91 – 7.60 | 8.50 – 7.73 |
| Amantadine | 0.391 | 100 | 9 | 2 | 9.05 – 7.37 | 9.05 – 7.48 |
| Atenolol | 100 | 100 | limit test | NA | NA | 7.38 – 7.63 |
| Bezafibrate | 9.8 | 100 | 3 | 3.2 | 7.83 – 7.59 | 7.10 – 7.60 |
| Carbamazepine | 100 | 100 | limit test | NA | NA | 7.37 – 7.60 |
| Chloridazon | 100 | 100 | limit test | NA | NA | 7.41 – 7.59 |
| Clothianidin | 0.000001 | 100 | 9 | 10 | 7.12 – 7.41 | 7.12 – 7.32 |
| DEET | 9.8 | 100 | 3 | 3.2 | 7.83 – 7.66 | 7.16 – 7.67 |
| Diclofenac | 1 | 100 | 6 | 2.5 | 7.96 – 7.57 | 7.17 – 7.41 |
| Flufenacet | 100 | 100 | limit test | NA | NA | 7.03 – 7.66 |
| Flumequine | 1 | 100 | 6 | 2.5 | 7.78 – 7.84 | 6.91 – 7.69 |
| Imidacloprid | 0.015 | 100 | 9 | 3 | 7.33 – 7.29 | 7.33 – 7.39 |
| Mecoprop | 100 | 100 | limit test | NA | NA | 7.00 – 7.62 |
| Metoprolol | 9.8 | 100 | 3 | 3.2 | 7.96 – 7.69 | 7.14 – 7.70 |
| Moxifloxacin | 100 | 100 | limit test | NA | NA | 6.91 – 7.52 |
| Oxytetracycline | 100 | 100 | limit test | NA | NA | 7.24 – 7.51 |
| Paracetamol | 1 | 100 | 6 | 2.5 | 7,55 – 7,41 | 7.41 – 7.46 |
| Pirimicarb | 9.8 | 100 | 3 | 3.2 | 7.88 – 7.62 | 7.29 – 7.62 |
| Sulfamethoxazole | 1 | 100 | 6 | 2.5 | 6.81 – 7.41 | 6.81 – 7.40 |
| Thiacloprid | 0.015 | 100 | 9 | 3 | 7.37 – 7.33 | 7.37 – 7.41 |
| Thiamethoxam | 0.015 | 100 | 9 | 3 | 7.33 – 7.30 | 7.33 – 7.37 |
| Venlafaxine | 1 | 100 | 6 | 2.5 | 7.57 – 7.58 | 6.99 – 7.58 |
| Zidovudine | 100 | 100 | limit test | NA | NA | 7.15 – 7.64 |

**Table A4 Acute $EC_{50}$ (mg $L^{-1}$) values for substances other than the neonicotinoid insecticides including their confidence intervals (CI) based on measured concentrations. NA depicts not applicable. Values with the prefix ">" (higher than) refer to the observation of mortality of less than 50% of test organisms at the indicated concentration.**

| | 24h | CI | 48h | CI | 72h | CI | 96h | CI |
|---|---|---|---|---|---|---|---|---|
| Alachlor | 31 | 30.6 – 31.0 | 20 | 19 – 21 | 23 | 21 – 26 | 12 | 11 – 13 |
| Amantadine | 14 | 9 – 19 | 8 | 6.4 – 10 | 6.3 | 5.1 – 7.5 | 4.8 | 3.0 – 6.6 |
| Atenolol | >75 | NA | >75 | NA | >75 | NA | >75 | NA |
| Bezafibrate | >61 | NA | >61 | NA | >61 | NA | >61 | NA |
| Carbamazepine | >113 | NA | >113 | NA | >113 | NA | >113 | NA |
| Chloridazon | >120 | NA | >120 | NA | >120 | NA | >120 | NA |
| DEET | >73 | NA | >73 | NA | >37 | NA | >37 | NA |
| Diclofenac | 42 | 31 – 53 | 41 | 27 – 55 | 26 | 24 – 29 | 21 | 19 – 23 |
| Flufenacet | >76 | NA | >76 | NA | >76 | NA | >76 | NA |
| Flumequine | >143 | NA | >143 | NA | >143 | NA | >143 | NA |
| Mecoprop | >89 | NA | >89 | NA | >89 | NA | >89 | NA |
| Metoprolol | >75 | NA | >75 | NA | >75 | NA | >75 | NA |
| Moxifloxacin | >101 | NA | >101 | NA | >101 | NA | >101 | NA |
| Oxytetracycline | >65 | NA | >65 | NA | >65 | NA | >65 | NA |
| Paracetamol | >72 | NA | >72 | NA | >72 | NA | >72 | NA |
| Pirimicarb | >95 | NA | >95 | NA | >95 | NA | >95 | NA |
| Sulfamethoxazole | >125 | NA | >125 | NA | >125 | NA | >125 | NA |
| Venlafaxine | >104 | NA | >69 | NA | 72 | 62 – 83 | 37 | 26 – 48 |
| Zidovudine | >151 | NA | >151 | NA | >151 | NA | >151 | NA |

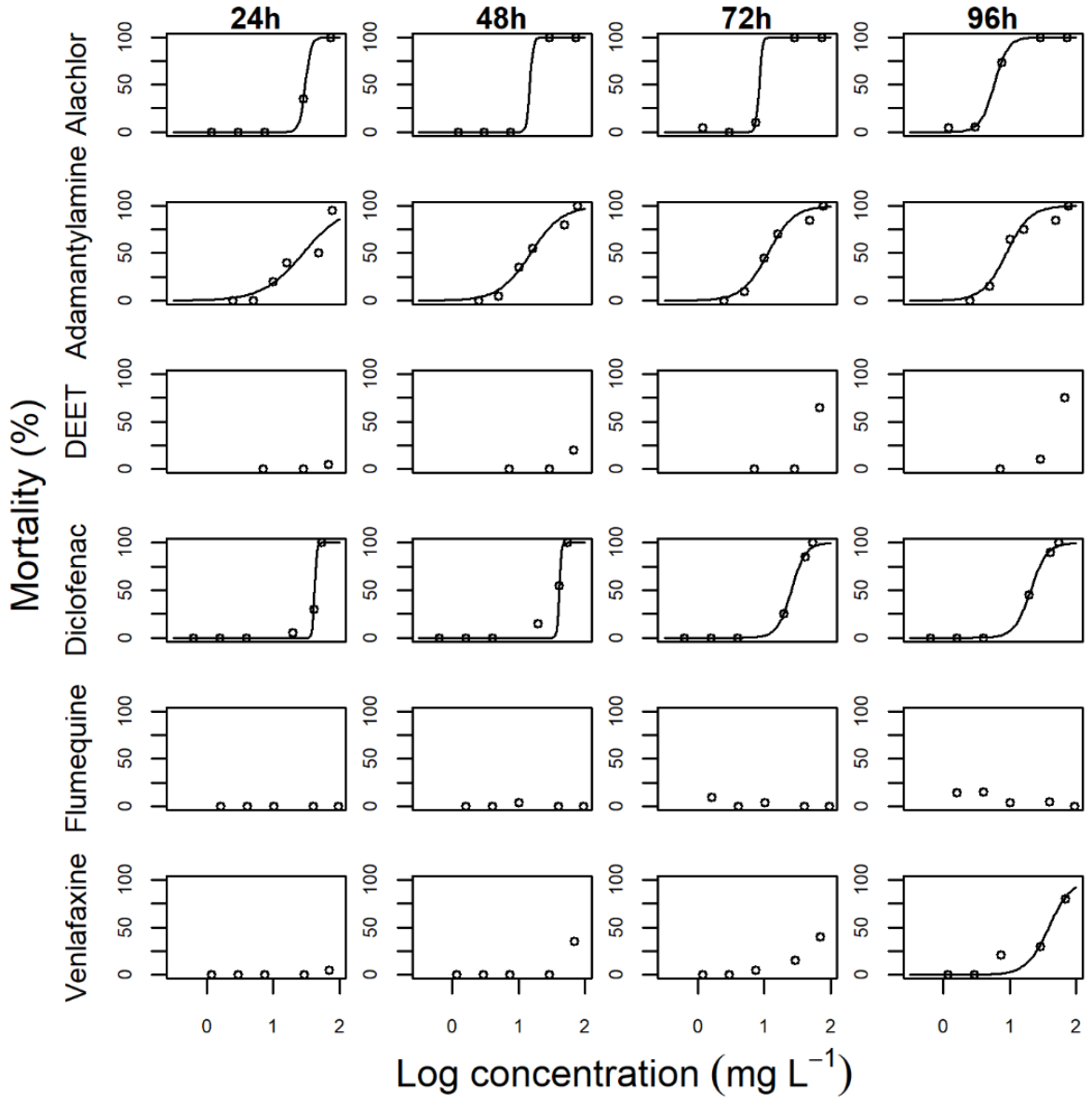


**Figure A3 Concentration-response data and curves for Nitocra spinipes exposed to 6 chemicals of emerging concern, recorded daily for 96h. Black circles show the mean observed mortality of quadruplicates in percent. Lines are fitted log-logistic dose-response models.**

### A2.3 Chemical analysis

A detailed overview of the results of the chemical analysis of all acute copepod tests and larval development tests is given in Table A5 and Table A6, respectively.

**Table A5 Overview of the results of the chemical analysis performed for the confirmation of test concentrations in all acute copepod tests. All concentrations are indicated in µg $L^{-1}$. NA stands for not applicable and refers to concentration treatments (CT) that were not tested for different substances. ND described no detection in the control treatments.**

| Substance | Stock solution | CT1 | CT2 | CT3 | CT4 | CT5 | CT6 | CT7 | CT8 | CT9 | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Alachlor | 105048 | 104447 | 73393 | 28746 | 14459 | 7273 | 3658 | NA | NA | NA | ND |
| Amantadine | NA | 36246 | 18308 | 9774 | 5074 | 2634 | 1367 | 730 | 365 | 182 | 0.026 |
| Atenolol | 81508 | 75268 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Bezafibrate | 62381 | 60982 | 32579 | 12656 | 5693 | 2561 | 1152 | NA | NA | NA | ND |
| Carbamazepine | 86694 | 113045 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Chloridazon | 119296 | 120455 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Clohtianidin | NA | 71761 | 11709 | 1907 | 311 | 51 | 8.3 | 1.3 | 0.22 | 0.036 | ND |
| DEET | 65655 | 72939 | 36720 | 12125 | NA | NA | NA | NA | NA | NA | ND |
| Diclofenac | 65922 | 54060 | 41140 | 19560 | 11448 | 6700 | 3921 | NA | NA | NA | ND |
| Flufenacet | 92237 | 75645 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Flumequine | 136654 | 143133 | 95118 | 39716 | 20369 | 10446 | 5357 | NA | NA | NA | ND |
| Imidacloprid | NA | 131542 | 11054 | 1050 | 100 | 8.9 | 0.94 | 0.15 | 0 | 0 | ND |
| Mecoprop | 86449 | 88511 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Metoprolol | 70203 | 74985 | 41036 | 13737 | NA | NA | NA | NA | NA | NA | ND |
| Moxifloxacin | 85621 | 101291 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Oxytetracycline | 64699 | 65043 | NA | NA | NA | NA | NA | NA | NA | NA | ND |
| Paracetamol | NA | 72321 | 41192 | 16649 | 7872 | 3722 | 1760 | NA | NA | NA | 0.16 |
| Pirimicarb | 89706 | 94543 | 48323 | 17198 | NA | NA | NA | NA | NA | NA | ND |
| Sulfamethoxazole | NA | 124835 | 52360 | 21738 | 9071 | 3785 | 1580 | NA | NA | NA | ND |
| Thiacloprid | NA | 82511 | 9074 | 860 | 83 | 7.1 | 0.83 | 0.16 | 0.065 | 0 | 0.0019 |
| Thiamethoxam | NA | 141601 | 23659 | 9051 | 2105 | 490 | 114 | 26 | 6.2 | 1.4 | ND |
| Venlafaxine | 93338 | 104258 | 69480 | 29010 | 14894 | 7646 | 3926 | NA | NA | NA | ND |
| Zidovudine | 146172 | 150798 | NA | NA | NA | NA | NA | NA | NA | NA | ND |

**Table A6 Overview of the results of the chemical analysis performed for the confirmation of test concentrations in all larval development tests. All concentrations are indicated in µg $L^{-1}$. NA stands for not applicable and refers to concentration treatments that were not tested for different substances. ND described no detection in the control treatments.**

| | CT1 | CT2 | CT3 | CT4 | CT5 | CT6 | Control |
|---|---|---|---|---|---|---|---|
| **Clothianidin** | | | | | | | |
| Day 0 | 14 | 2.9 | 0.79 | 0.25 | 0.081 | 0.032 | 0 |
| Day 4 | 16 | 2.5 | 0.79 | 0.25 | 0.081 | 0.033 | 0.018 |
| Day 7 | 13 | 2.3 | 0.83 | 0.28 | 0.078 | 0.027 | 0.0080 |
| **Imidacloprid** | | | | | | | |
| Day 0 | 115 | 36 | 13 | 4.1 | 1.6 | 0.45 | 0 |
| Day 4 | 115 | 31 | 12 | 4.2 | 2.0 | 0.43 | 0.0063 |
| Day 7 | 99 | 38 | 13 | 4.2 | 0.85 | 0.47 | 0.04 |
| **Thiacloprid** | | | | | | | |
| Day 0 | 94 | 33 | 8.3 | 3.0 | 0.77 | 0.63 | 0.0018 |
| Day 4 | 92 | 31 | 8.1 | 2.9 | 0.88 | 0.36 | 0.00069 |
| Day 7 | 96 | 31 | 9.5 | 2.2 | 0.92 | 0.38 | 0.00080 |
| **Thiamethoxam** | | | | | | | |
| Day 0 | 100 | 31 | 11 | 3.6 | 1.2 | 0.31 | 0 |
| Day 4 | 98 | 31 | 11 | 3.5 | 1.5 | 0.31 | 0 |
| Day 7 | 100 | 31 | 11 | 3.6 | 0.7 | 0.33 | 0 |

### A2.4 Database exports

The database exports including all data considered for the EQS derivation for the four neonicotinoid insecticides can be consulted on the Marine Data Archive via the following link: http://mda.vliz.be/directlink.php?fid=VLIZ_00000535_5dd3eba4c6c5b.

## A3 Discussion

### A3.1 Data reliability

Data reliability of the OPP database was high due to the requirements for implementation into the database being a thorough revision by ecotoxicological experts at US EPA. The highest uncertainty for data retrieved from the ECOTOX database was linked to the status of chemical analysis. Many data was labeled as *not coded* which presumably is a relic of merging several former EPA databases. Unfortunately, the link to the initial reference was not available in the database extract and due to the relatively high amount of data with *not coded* chemical analysis status, we decided to include the data in our final dataset. For the data labeled with *not measured* chemical analysis, we checked the initial publication if available to confirm the database status. If against the indication in the database, chemical analysis to confirm contaminant concentrations in the test(s) was performed, we included the data in our database otherwise it was excluded; The only exception was made where the remaining data did not fulfill the basic dataset (algae, crustacean and fish) for EQS derivation. This was the case only once for a short-term $EC_{50}$ value for *Procambarus clarcii* exposed to TMX that was included as the lowest available crustacean $EC_{50}$ but not the lowest acute endpoint overall.

Hence, while the OPP database includes highly reliable data due to revision by US EPA experts, the data reliability of the ECOTOX database is more difficult to assess. This is due to the fact that there is no thorough revision of the data before inclusion. Next, data sources are not always indicated making individual reliability checking difficult.

### A3.2 Assumptions and justifications for the EQS derivation

Given the fact that neonicotinoid insecticides have a known mode of action [93] and insects have been found to be the most sensitive taxonomic group [97], the MAC-EQS for CLO was derived using an AF of 10 based on the availability of short-term $L(E)C_{50}$ values for freshwater algae, crustacean, fish and insects. Additionally, $L(E)C_{50}$ values for saltwater algae, crustacean, fish and mollusks were available, where *Crassostrea virginica* and *N. spinipes* were considered as additional marine taxonomic groups. *N. spinipes* was evaluated as an additional marine taxonomic group because of its different life form and feeding strategy (benthic primary consumer) as compared to the freshwater crustacean *D. magna* (mainly pelagic filter-feeder). Considering that the mode of action of CLO is known and a representative species of the most sensitive taxonomic group (insects, 96h-$EC_{50}$ for *Chironomus dilutus*) was available, an AF of 10 was applied. The AA-EQS for CLO was derived with an AF of 10 for freshwater and 50 for saltwater environments. This was based on the availability of long-term results for freshwater algae, crustacean, fish, aquatic plants and insects and long-term results for saltwater algae and crustacean where *Americamysis bahia* was considered as additional marine taxonomic group. *A. bahia* is a benthic predator feeding on smaller crustacean or algae clearly differing from e.g. *D. magna*. .

The MAC-EQS for IMI was derived using an AF of 10 based on the availability of short-term $L(E)C_{50}$ of algae, crustacean, fish, insects and mollusks where *C. virginica* and *Americamysis bahia* were considered as additional marine taxonomic groups. The AA-EQS for IMI was derived with an AF of 10 and 50 for the freshwater and the saltwater environment, respectively. This was based on long-term data available for freshwater algae, crustacean, fish, aquatic plants and mollusks and *A. bahia* representing an additional marine taxonomic group.

For TCP the MAC-EQS was derived using an AF of 10 for both environments based on short-term data availability for algae, crustacean, fish, insects and mollusks. Short-term data for *C. virginica* and *N. spinipes* was considered as data for additional marine taxonomic groups. The AA-EQS for TCP was derived using an AF of 10 and 50 for the freshwater and saltwater environment; respectively. Long-term data was available for algae, crustacean, fish and insects, whereof *N. spinipes* was considered as representative of an additional marine taxonomic group.

The MAC-EQS for TMX was derived using an AF of 10 for both environments based on data available for algae, crustacean, fish, aquatic plants, insects and mollusks. *C. virginica* and *N. spinipes* were considered as representatives of an additional marine taxonomic group with available short-term data. The AA-EQS was derived using an AF of 10 and 50 for the freshwater and saltwater environment, respectively. This was based on long-term data availability for algae, crustacean, fish, aquatic plants and insects where *N. spinipes* was considered as additional marine taxonomic group.

### A3.3 Reference threshold data for neonicotinoid insecticides

Table A7 contains reference threshold data for neonicotinoid insecticides.

**Table A7 Reference threshold data (µg $L^{-1}$) for neonicotinoid insecticides in freshwater environments.**

| Substance | Smit et al.[a] | | Canada[b] | | European Commission (Joint Research Center)[c] | | United States[d] | |
|---|---|---|---|---|---|---|---|---|
| | Acute | Chronic | Acute | Chronic | Acute | Chronic | Acute | Chronic |
| Clothianidin | NA[e] | NA | 1.5 | 0.0015 | NA | 0.13 | 11 | 0.05 |
| Imidacloprid | 0.2 | 0.0083 | 0.36 | 0.041 | NA | 0.0083 | 0.39 | 0.01 |
| Thiacloprid | NA | NA | NA | NA | NA | 0.01 | 19 | 0.97 |
| Thiamethoxam | NA | NA | 9 | 0.026 | NA | 0.042 | 18 | 0.74 |

[a] Environmental Quality Standard (EQS) [104]

[b] [105, 178, 179]

[c] Predicted no-effect concentrations (PNEC) [72]

[d] Life benchmark for aquatic invertebrates [103]

[e] NA = Not Available

# Annex B

Supportive information to Chapter 5

# Annex B

**Table B1 Overview of the quantity and type of ecotoxicity data available and used for automated PNEC derivation.**

| Substance | CAS number | Most sensitive species | Species group of most sensitive species | Lowest effect concentration (µg/L) | Endpoint | Test duration (d) | Effect measurement | Number of acute endpoints | Number of chronic endpoints | AF | PNEC (ng/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Atrazine | 1912249 | Elodea canadensis | Plants | 4.8E-10 | $EC_{10}$ | 28 | Weight | 82 | 177 | 10 | 0.000000048 |
| 17alpha-Ethinylestradiol | 57636 | Rutilus rutilus | Fish | 0.000040 | NOEC | 518 | Weight | 7 | 52 | 50 | 0.00080 |
| 17beta-Trenbolone | 10161338 | Pimephales promelas | Fish | 0.0015 | NOEC | 21 | Sexual development | 0 | 9 | 1000 | 0.0015 |
| Estrone | 53167 | Oryzias latipes | Fish | 0.0050 | NOEC | 15 | Hatch | 0 | 5 | 1000 | 0.0050 |
| Imidacloprid | 138261413 | Daphnia magna | Crustaceans | 0.00030 | NOEC | 6 | Length | 41 | 27 | 50 | 0.0060 |
| Testosterone | 58220 | Lymnaea stagnalis | Molluscs | 0.0068 | NOEC | 21 | Clutch production | 3 | 3 | 1000 | 0.0068 |
| 17beta-Estradiol | 50282 | Oncorhynchus mykiss | Fish | 0.00042 | NOEC | 50 | Sperm cell counts | 9 | 35 | 50 | 0.0084 |
| Amitriptyline hydrochloride | 549188 | Danio rerio | Fish | 0.010 | NOEC | 5 | Length | 1 | 1 | 1000 | 0.010 |
| Melengestrol acetate | 2919666 | Xenopus laevis | Amphibians | 0.010 | NOEC | 60 | Weight | 0 | 1 | 1000 | 0.010 |
| Terbutryn | 886500 | Fragilaria capucina ssp. rumpens | Algae | 0.015 | $EC_{10}$ | 4 | Chlorophyll A concentration | 11 | 16 | 1000 | 0.015 |
| 17-Methyltestosterone | 58184 | Marisa cornuarietis | Molluscs | 0.030 | NOEC | 150 | Imposex. intersex conditions | 1 | 9 | 1000 | 0.030 |
| Chlorfenvinphos | 470906 | Ceriodaphnia dubia | Crustaceans | 0.40 | $LC_{50}$ | 2 | Mortality | 18 | 0 | 10000 | 0.040 |
| Estriol | 50271 | Oryzias latipes | Fish | 0.047 | NOEC | 15 | Hatch | 0 | 2 | 1000 | 0.047 |
| Diuron | 330541 | Chara vulgaris | Algae | 0.00050 | NOEC | 14 | Growth rate | 41 | 62 | 10 | 0.050 |
| 5alpha-Dihydrotestosterone | 521186 | Danio rerio | Fish | 0.10 | NOEC | 60 | Stage | 0 | 3 | 1000 | 0.10 |
| beta-Sitosterol | 83465 | Lymnaea stagnalis | Molluscs | 0.10 | NOEC | 56 | Clutch production | 0 | 5 | 1000 | 0.10 |
| Clothianidin | 210880925 | Planorbella pilsbryi | Molluscs | 0.10 | $EC_{10}$ | 28 | Weight | 6 | 3 | 1000 | 0.10 |
| Cybutryne | 28159980 | Ulnaria ulna | Algae | 0.0018 | $EC_{10}$ | 4 | Chlorophyll A concentration | 21 | 46 | 10 | 0.18 |
| Isoproturon | 34123596 | Fragilaria crotonensis | Algae | 0.21 | $EC_{10}$ | 4 | Chlorophyll A concentration | 1 | 27 | 1000 | 0.21 |
| Linuron | 330552 | Marisa cornuarietis | Molluscs | 0.030 | NOEC | 152.2 | Sexual development | 8 | 54 | 100 | 0.30 |
| Norethindrone | 68224 | Pimephales promelas | Fish | 0.37 | NOEC | 28 | Weight | 0 | 4 | 1000 | 0.37 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flufenacet | 142459583 | Lemna gibba | Plants | 0.44 | NOEL | 14 | Abundance | 5 | 5 | 1000 | 0.44 |
| Fluoxetine hydrochloride | 56296787 | Pimephales promelas | Fish | 0.028 | NOEC | 21 | Sperm cell counts | 9 | 19 | 50 | 0.56 |
| Mestanolone | 521119 | Pimephales promelas | Fish | 0.70 | NOEC | 114 | Size | 0 | 2 | 1000 | 0.70 |
| Dichlorvos | 62737 | Daphnia magna | Crustaceans | 0.085 | $LC_{50}$ | 2 | Mortality | 101 | 7 | 100 | 0.85 |
| Bisphenol A | 80057 | Mytilus galloprovincialis | Molluscs | 0.010 | NOEC | 2 | Normal | 19 | 31 | 10 | 1.0 |
| Bezafibrate | 41859670 | Mytilus galloprovincialis | Molluscs | 1.0 | NOEC | 2 | Normal | 1 | 4 | 1000 | 1.0 |
| Indomethacin | 53861 | Danio rerio | Fish | 1.0 | NOEC | 16 | Number spawning | 2 | 2 | 1000 | 1.0 |
| Pentachlorophenol | 87865 | Daphnia magna | Crustaceans | 0.053 | NOEC | 21 | Length | 152 | 32 | 50 | 1.1 |
| S-Metolachlor | 87392129 | Pseudokirchneriella subcapitata | Algae | 1.5 | NOEL | 5 | Abundance | 5 | 8 | 1000 | 1.5 |
| Pirimicarb | 23103982 | Daphnia pulex | Crustaceans | 19 | $LC_{50}$ | 2 | Mortality | 6 | 1 | 10000 | 1.9 |
| Di(2-ethylhexyl) phthalate | 117817 | Danio rerio | Fish | 2.0 | NOEC | 21 | Fully developed oocytes | 4 | 10 | 1000 | 2.0 |
| Thiacloprid | 111988499 | Baetis rhodani | Insects | 0.31 | NOEC | 2 | Drift | 13 | 6 | 100 | 3.1 |
| Oxytetracycline hydrochloride | 2058460 | Anabaena cylindrica | Algae | 3.1 | NOEC | 6 | Abundance | 4 | 21 | 1000 | 3.1 |
| 2-Hydroxy-4-methoxybenzophenone | 131577 | Isochrysis galbana | Algae | 3.7 | $EC_{10}$ | 3 | Population growth rate | 1 | 5 | 1000 | 3.7 |
| Dexamethasone | 50022 | Xenopus laevis | Amphibians | 3.9 | NOEC | 7 | Length | 0 | 3 | 1000 | 3.9 |
| Triclosan | 3380345 | Pseudokirchneriella subcapitata | Algae | 0.20 | NOEC | 3 | Biomass | 9 | 19 | 50 | 4.0 |
| Ibuprofen | 15687271 | Mytilus galloprovincialis | Molluscs | 0.25 | NOEC | 15 | Condition index | 3 | 16 | 50 | 5.0 |
| Acetamiprid | 135410207 | Simulium latigonium | Insects | 0.50 | NOEC | 2 | Drift | 8 | 6 | 100 | 5.0 |
| Tetracycline | 60548 | Gambusia holbrooki | Fish | 0.50 | NOEC | 4 | Length | 5 | 6 | 100 | 5.0 |
| DEET | 134623 | Pimephales promelas | Fish | 0.60 | NOEC | 2 | Organ weight in relationship to body weight | 5 | 3 | 100 | 6.0 |
| Terbutylazine | 5915413 | Pseudokirchneriella subcapitata | Algae | 0.60 | NOEL | 5 | Abundance | 8 | 13 | 100 | 6.0 |
| Quinoxyfen | 124495187 | Pseudokirchneriella subcapitata | Algae | 6.4 | NOEL | 5 | Abundance | 3 | 4 | 1000 | 6.4 |
| Clofibric acid | 882097 | Oncorhynchus mykiss | Fish | 0.97 | NOEC | 28 | Weight | 4 | 10 | 100 | 9.7 |
| Acetaminophen | 103902 | Danio rerio | Fish | 1.0 | NOEC | 5 | Weight | 7 | 9 | 100 | 10 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sodium diclofenac | 15307796 | Danio rerio | Fish | 10 | NOEC | 14 | Progeny counts/numbers | 2 | 8 | 1000 | 10 |
| Diethylstilbestrol | 56531 | Daphnia magna | Crustaceans | 10 | NOEC | 6 | Size | 3 | 4 | 1000 | 10 |
| Levofloxacin | 100986854 | Lemna gibba | Plants | 10 | NOEC | 7 | Biomass | 0 | 3 | 1000 | 10 |
| Dimethoate | 60515 | Chironomus dilutus | Insects | 1.3 | $LC_{50}$ | 4 | Mortality | 73 | 7 | 100 | 13 |
| Sulfamethoxazole | 723466 | Caenorhabditis elegans | Worms | 1.3 | $EC_{10}$ | 4 | Length | 4 | 6 | 100 | 13 |
| Carbamazepine | 298464 | Stenonema sp. | Insects | 0.20 | NOEC | 9 | Molting | 4 | 33 | 10 | 20 |
| Naproxen | 22204531 | Limnodynastes peronii | Amphibians | 10 | NOEC | 21 | Stage | 3 | 1 | 500 | 20 |
| Atenolol | 29122687 | Microcystis aeruginosa | Algae | 20 | NOEC | 3 | Chlorophyll A concentration | 1 | 4 | 1000 | 20 |
| Thiamethoxam | 153719234 | Planorbella pilsbryi | Molluscs | 21 | $EC_{10}$ | 28 | Weight | 8 | 3 | 1000 | 21 |
| 2,4-Dichlorophenoxyacetic acid | 94757 | Myriophyllum sibiricum | Plants | 2.0 | NOEC | 14 | Number of roots | 49 | 51 | 50 | 40 |
| Dibutyl phthalate | 84742 | Spirodela polyrrhiza | Plants | 5.0 | NOEC | 7 | Quantity | 15 | 11 | 100 | 50 |
| Paroxetine | 61869087 | Ceriodaphnia dubia | Crustaceans | 580 | $LC_{50}$ | 2 | Mortality | 1 | 2 | 10000 | 58 |
| Diisodecyl phthalate | 26761400 | Daphnia magna | Crustaceans | 60 | NOEC | 21 | Reproduction. general | 0 | 1 | 1000 | 60 |
| Dihexyl phthalate | 84753 | Daphnia magna | Crustaceans | 84 | NOEC | 21 | Reproduction. general | 1 | 2 | 1000 | 84 |
| Aspirin | 50782 | Danio rerio | Fish | 100 | NOEC | 7 | Progeny counts/numbers | 1 | 3 | 1000 | 100 |
| Ciprofloxacin | 85721331 | Lemna gibba | Plants | 100 | NOEC | 7 | Biomass | 1 | 1 | 1000 | 100 |
| Hydrocortisone | 50237 | Daphnia magna | Crustaceans | 100 | NOEC | 6 | Progeny counts/numbers | 0 | 2 | 1000 | 100 |
| Progesterone | 57830 | Daphnia magna | Crustaceans | 100 | NOEC | 9 | Sex ratio | 0 | 1 | 1000 | 100 |
| Stigmasterol | 83487 | Gambusia sp. | Fish | 100 | NOEC | 28 | Length | 0 | 1 | 1000 | 100 |
| Methiocarb | 2032657 | Pteronarcys californica | Insects | 5.4 | $LC_{50}$ | 4 | Mortality | 15 | 3 | 50 | 110 |
| Chloridazon | 1698608 | Skeletonema costatum | Algae | 108 | NOEL | 5 | Abundance | 0 | 6 | 1000 | 110 |
| Dinoseb | 88857 | Pimephales promelas | Fish | 15 | NOEC | 64 | Length | 15 | 4 | 100 | 150 |
| Trimethoprim | 738705 | Danio rerio | Fish | 157 | NOEC | 21 | Weight | 2 | 13 | 1000 | 160 |
| Piperonyl butoxide | 51036 | Hyalella azteca | Crustaceans | 25 | NOEC | 10 | Weight | 23 | 6 | 100 | 250 |
| Diazepam | 439145 | Danio rerio | Fish | 291 | NOEC | 14 | Condition index | 2 | 1 | 1000 | 290 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4-Hydroxyandrostenedione | 566483 | Nucella lapillus | Molluscs | 300 | NOEC | 91.32 | Imposex. intersex conditions | 0 | 1 | 1000 | 300 |
| Sulfamethazine | 57681 | Lemna gibba | Plants | 300 | NOEC | 7 | Biomass | 1 | 3 | 1000 | 300 |
| Propranolol hydrochloride | 4199104 | Daphnia magna | Crustaceans | 400 | NOEC | 21 | Progeny counts/numbers | 1 | 1 | 1000 | 400 |
| Flumequine | 42835256 | Arbacia lixula | Sea Urchins | 500 | NOEC | 3 | Abnormal | 0 | 4 | 1000 | 500 |
| Benzyl butyl phthalate | 85687 | Pimephales promelas | Fish | 65 | NOEL | 19 | Hatch | 14 | 5 | 100 | 650 |
| Trenbolone acetate | 10161349 | Pomoxis nigromaculatus | Fish | 1000 | NOEL | 26 | Length | 0 | 1 | 1000 | 1000 |
| Salicylic acid | 69727 | Pseudokirchneriella subcapitata | Algae | 1790 | NOEC | 2 | Abundance | 1 | 4 | 1000 | 1800 |
| 4-Androstene-3,17-dione | 63058 | Daphnia magna | Crustaceans | 2291 | NOEC | 21 | Abnormal | 1 | 1 | 1000 | 2300 |
| Cortisone | 53065 | Daphnia magna | Crustaceans | 2884 | NOEC | 21 | Progeny counts/numbers | 0 | 1 | 1000 | 2900 |
| Dimethyl phthalate | 131113 | Cyprinodon variegatus | Fish | 29000 | $LC_{50}$ | 4 | Mortality | 8 | 1 | 10000 | 2900 |
| Diethyl phthalate | 84662 | Danio rerio | Fish | 427 | NOEC | 38.333 | Hatch | 12 | 6 | 100 | 4300 |
| Dipropyl phthalate | 131168 | Xenopus laevis | Amphibians | 5000 | NOEC | 4 | Deformation | 1 | 1 | 1000 | 5000 |
| Enrofloxacin | 93106606 | Daphnia magna | Crustaceans | 5000 | NOEC | 21 | Time to first progeny | 1 | 1 | 1000 | 5000 |
| Ethylparaben | 120478 | Pseudokirchneriella subcapitata | Algae | 5000 | NOEC | 3 | Population growth rate | 1 | 1 | 1000 | 5000 |
| Medroxyprogesterone | 520854 | Ceriodaphnia dubia | Crustaceans | 5000 | NOEC | 7 | Progeny counts/numbers | 0 | 1 | 1000 | 5000 |
| Methylparaben | 99763 | Pseudokirchneriella subcapitata | Algae | 5000 | NOEC | 3 | Population growth rate | 1 | 3 | 1000 | 5000 |
| Chloramphenicol | 56757 | Tetraselmis suecica | Algae | 10000 | NOEC | 4 | Abundance | 3 | 1 | 1000 | 10000 |
| Metronidazole | 443481 | Americamysis bahia | Crustaceans | 182000 | $LC_{50}$ | 4 | Mortality | 1 | 1 | 10000 | 18000 |

**Table B2 Summary of predicted no-effect concentrations (PNEC) and grab sample-based risk quotients (RQ) for all target substances. The PNEC is given in ng $L^{-1}$. # detects indicates the number of detects per substance across all sampling locations and across SC2-SC5. $RQ_{median}$ indicates the median RQ for all detects and $RQ_{max.}$ the highest observed RQ. The total amount of grab samples taken was 16 across all sampling campaigns and locations. Data is listed per substance group arranged with descending $RQ_{median}$.**

| Class | Substance | CAS-number | PNEC | $RQ_{median}$ | $RQ_{max.}$ | # detects |
|---|---|---|---|---|---|---|
| PCP | 2-Hydroxy-4-methoxybenzophenone | 131577 | 3.7 | 1.7 | 2.2 | 4 |
| PCP | Ethylparaben | 120478 | 5000 | 1.7 | 2.2 | 1 |
| PCP | DEET | 134623 | 6.0 | 0.98 | 2.2 | 16 |
| PCP | Piperonyl butoxide | 51036 | 250 | 0.0072 | 0.051 | 8 |
| PCP | Methylparaben | 99763 | 5000 | 0.00088 | 0.12 | 10 |
| Pesticides | Imidacloprid | 138261413 | 0.0060 | 375 | 1243 | 16 |
| Pesticides | Acetamiprid | 135410207 | 5.0 | 341 | 1243 | 2 |
| Pesticides | Terbutryn | 886500 | 7[a] | 33 | 96 | 6 |
| Pesticides | Clothianidin | 210880925 | 0.10[b] | 8.0 | 35 | 14 |
| Pesticides | Linuron | 330552 | 0.30 | 6.6 | 207 | 10 |
| Pesticides | Flufenacet | 142459583 | 0.44 | 4.2 | 109 | 13 |
| Pesticides | S-Metolachlor | 87392129 | 1.5 | 2.0 | 5.6 | 15 |
| Pesticides | Thiacloprid | 111988499 | 3.1[b] | 0.30 | 11 | 15 |
| Pesticides | Pirimicarb | 23103982 | 1.9 | 0.23 | 7.3 | 11 |
| Pesticides | Terbutylazine | 5915413 | 6 | 0.18 | 0.22 | 16 |
| Pesticides | Cybutryne | 28159980 | 3[a] | 0.12 | 0.40 | 12 |
| Pesticides | Thiamethoxam | 153719234 | 42[b] | 0.095 | 2.5 | 12 |
| Pesticides | 2,4-Dichlorophenoxyacetic acid | 94757 | 40 | 0.047 | 0.21 | 10 |
| Pesticides | Dinoseb | 88857 | 145 | 0.051 | 0.21 | 4 |
| Pesticides | Pentachlorophenol | 87865 | 400[a] | 0.031 | 0.047 | 7 |
| Pesticides | Chloridazon | 1698608 | 108 | 0.029 | 0.20 | 16 |
| Pesticides | Diuron | 330541 | 200[a] | 0.019 | 0.062 | 13 |
| Pesticides | Isoproturon | 34123596 | 300[a] | 0.010 | 0.14 | 16 |
| Pesticides | Atrazine | 1912249 | 600[a] | 0.0023 | 0.0060 | 16 |

| Class | Substance | CAS-number | PNEC | $RQ_{median}$ | $RQ_{max.}$ | # detects |
|---|---|---|---|---|---|---|
| Pesticides | Dimethoate | 60515 | 13 | 0.0010 | 28 | 2 |
| Pharmaceuticals | Bezafibrate | 41859670 | 1.0 | 3.1 | 6.9 | 14 |
| Pharmaceuticals | Sodium diclofenac | 15307796 | 10 | 2.5 | 8.4 | 11 |
| Pharmaceuticals | Acetaminophen | 103902 | 10 | 0.68 | 12 | 15 |
| Pharmaceuticals | Carbamazepine | 298464 | 20 | 0.61 | 1.6 | 16 |
| Pharmaceuticals | Clarithromycin | 81103119 | 80[c] | 0.061 | 0.079 | 4 |
| Pharmaceuticals | Atenolol | 29122687 | 20 | 0.30 | 2.9 | 16 |
| Pharmaceuticals | Naproxen | 22204531 | 200 | 0.28 | 1.0 | 4 |
| Pharmaceuticals | Flumequine | 42835256 | 250[c] | 0.0085 | 0.048 | 3 |
| Pharmaceuticals | Metronidazole | 443481 | 130[c] | 0.0074 | 0.034 | 8 |
| Pharmaceuticals | Sulfamethoxazole | 723466 | 600[c] | 0.0059 | 0.024 | 16 |
| Pharmaceuticals | Propranolol hydrochloride | 4199104 | 400 | 0.0044 | 0.022 | 15 |
| Pharmaceuticals | Sulfamethazine | 57681 | 300 | 0.0043 | 0.15 | 6 |
| Pharmaceuticals | Nalidixic acid | 389082 | 16000[c] | 0.00039 | 0.00060 | 5 |
| Pharmaceuticals | Diazepam | 439145 | 291 | 0.00020 | 0.00062 | 5 |
| Pharmaceuticals | Trimethoprim | 738705 | 500[c] | 0.00086 | 0.0092 | 17 |
| Phenols | Bisphenol A | 80057 | 1.0 | 0.34 | 1413 | 5 |
| Phthalates | Dibutyl phthalate | 84742 | 50 | 9.0 | 54 | 15 |
| Phthalates | Diisodecyl phthalate | 26761400 | 60 | 1.9 | 7.3 | 4 |
| Phthalates | Dihexyl phthalate | 84753 | 84 | 0.32 | 1.8 | 7 |
| Phthalates | Di(2-ethylhexyl) phthalate | 117817 | 1300[a] | 0.22 | 0.31 | 14 |
| Phthalates | Benzyl butyl phthalate | 85687 | 646 | 0.13 | 0.45 | 15 |
| Phthalates | Diethyl phthalate | 84662 | 4272 | 0.11 | 41 | 10 |
| Steroids | 17alpha-Ethinylestradiol | 57636 | 0.00080[b] | 1125 | 3125 | 10 |
| Steroids | 17beta-Estradiol | 50282 | 0.0084[b] | 833 | 5560 | 9 |
| Steroids | 17beta-Trenbolone | 10161338 | 0.0015 | 517 | 2000 | 12 |
| Steroids | Estrone | 53167 | 0.0050[b] | 130 | 400 | 9 |
| Steroids | Testosterone | 58220 | 0.0068 | 44 | 515 | 9 |

| Class | Substance | CAS-number | PNEC | $RQ_{median}$ | $RQ_{max.}$ | # detects |
|---|---|---|---|---|---|---|
| Steroids | 17-Methyltestosterone | 58184 | 0.030 | 19 | 193 | 11 |
| Steroids | Mestanolone | 521119 | 0.70 | 18 | 94 | 7 |
| Steroids | 5alpha-Dihydrotestosterone | 521186 | 0.10 | 4.0 | 25 | 10 |
| Steroids | Norethindrone | 68224 | 0.37 | 2.0 | 28 | 6 |
| Steroids | Dexamethasone | 50022 | 3.9 | 1.9 | 14 | 8 |
| Steroids | Trenbolone acetate | 10161349 | 1000 | 0.17 | 3.0 | 6 |
| Steroids | Hydrocortisone | 50237 | 100 | 0.053 | 2131 | 8 |
| Steroids | Progesterone | 57830 | 100 | 0.042 | 193 | 10 |
| Steroids | Diethylstilbestrol | 56531 | 10 | 0.024 | 1.4 | 5 |
| Steroids | 4-Hydroxyandrostenedione | 566483 | 300 | 0.015 | 3125 | 10 |
| Steroids | Cortisone | 53065 | 2884 | 0.0029 | 380 | 10 |
| Steroids | 4-Androstene-3,17-dione | 63058 | 2291 | 0.0010 | 28 | 7 |
| Steroids | Medroxyprogesterone | 520854 | 5000 | 0.00015 | 20 | 10 |

**Table B3 Summary of predicted no-effect concentrations (PNEC) and passive sampling-based risk quotients (RQ) for all target substances. The PNEC is given in ng $L^{-1}$. # detects indicates the number of detects per substance across all sampling locations and across SC2-SC5. $RQ_{median}$ indicates the median RQ for all detects and $RQ_{max.}$ the highest observed RQ. The total amount of grab samples taken was 16 across all sampling campaigns and locations. Data is listed per substance group arranged with descending $RQ_{median}$.**

| Class | Substance | CAS-number | PNEC | $RQ_{median}$ | $RQ_{max.}$ | # detects |
|---|---|---|---|---|---|---|
| PCP | DEET | 134623 | 6.0 | 1.7 | 1.8 | 7 |
| PCP | Methylparaben | 99763 | 5000 | 0.00014 | 0.00014 | 1 |
| Pesticides | Imidacloprid | 138261413 | 0.0060 | 188 | 681 | 10 |
| Pesticides | Flufenacet | 142459583 | 0.44 | 5.7 | 13 | 10 |
| Pesticides | Linuron | 330552 | 0.30 | 2.8 | 18 | 10 |
| Pesticides | S-Metolachlor | 87392129 | 1.5 | 0.78 | 6.6 | 12 |
| Pesticides | Terbuthylazine | 5915413 | 6.0 | 0.38 | 3.1 | 12 |
| Pesticides | Terbutryn | 886500 | 6.5[a] | 0.18 | 0.26 | 4 |
| Pesticides | Pirimicarb | 23103982 | 1.9 | 0.10 | 0.25 | 8 |
| Pesticides | 2,4-Dichlorophenoxyacetic acid | 94757 | 40 | 0.082 | 0.17 | 9 |
| Pesticides | Cybutryne | 28159980 | 2.5[a] | 0.063 | 0.23 | 12 |
| Pesticides | Thiacloprid | 111988499 | 10[b] | 0.027 | 0.12 | 9 |
| Pesticides | Chloridazon | 1698608 | 108 | 0.026 | 0.11 | 12 |
| Pesticides | Thiamethoxam | 153719234 | 42[b] | 0.023 | 0.045 | 10 |
| Pesticides | Diuron | 330541 | 200[a] | 0.0076 | 0.018 | 12 |
| Pesticides | Isoproturon | 34123596 | 300[a] | 0.0066 | 0.048 | 12 |
| Pesticides | Clothianidin | 210880925 | 130[b] | 0.0062 | 0.021 | 8 |
| Pesticides | Atrazine | 1912249 | 600[a] | 0.0020 | 0.0052 | 11 |
| Pharmaceuticals | Bezafibrate | 41859670 | 1.0 | 8.5 | 24 | 10 |
| Pharmaceuticals | Amitriptyline hydrochloride | 549188 | 0.010 | 7.0 | 14 | 4 |
| Pharmaceuticals | Fluoxetine hydrochloride | 56296787 | 0.56 | 0.69 | 0.90 | 2 |
| Pharmaceuticals | Acetaminophen | 103902 | 10 | 0.56 | 6.4 | 10 |
| Pharmaceuticals | Azithromycin | 83905015 | 20[c] | 0.53 | 1.2 | 5 |
| Pharmaceuticals | Carbamazepine | 298464 | 20 | 0.20 | 0.46 | 12 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pharmaceuticals | Clarithromycin | 81103119 | 80[c] | 0.010 | 0.024 | 9 |
| Pharmaceuticals | Metronidazole | 443481 | 130[c] | 0.0045 | 0.0079 | 4 |
| Pharmaceuticals | Trimethoprim | 738705 | 500[c] | 0.00090 | 0.0042 | 12 |
| Pharmaceuticals | Flumequine | 42835256 | 250[c] | 0.00069 | 0.00075 | 3 |
| Pharmaceuticals | Diazepam | 439145 | 291 | 0.00046 | 0.00046 | 1 |
| Pharmaceuticals | Nalidixic acid | 389082 | 16000[c] | 0.000038 | 0.00021 | 6 |
| Phenols | Bisphenol A | 80057 | 1.0 | 33 | 55 | 8 |
| Phthalates | Dihexyl phthalate | 84753 | 84 | 1.1 | 1.1 | 1 |
| Phthalates | Dibutyl phthalate | 84742 | 50 | 0.52 | 0.86 | 10 |
| Phthalates | Diisodecyl phthalate | 26761400 | 60 | 0.22 | 0.90 | 10 |
| Phthalates | Benzyl butyl phthalate | 85687 | 646 | 0.0086 | 0.014 | 10 |
| Phthalates | Dimethyl phthalate | 131113 | 2900 | 0.0034 | 0.0036 | 3 |
| Phthalates | Di(2-ethylhexyl) phthalate | 117817 | 1300[a] | 0.0017 | 0.0029 | 9 |
| Steroids | 17beta-Estradiol | 50282 | 0.40[b] | 88 | 126 | 7 |
| Steroids | 17beta-Trenbolone | 10161338 | 0.0015 | 67 | 667 | 3 |
| Steroids | 17-Methyltestosterone | 58184 | 0.030 | 30 | 30 | 1 |
| Steroids | 5alpha-Dihydrotestosterone | 521186 | 0.10 | 13 | 146 | 5 |
| Steroids | 17alpha-Ethinylestradiol | 57636 | 0.035[b] | 11 | 11 | 1 |
| Steroids | Dexamethasone | 50022 | 3.9 | 0.66 | 2.2 | 8 |
| Steroids | Mestanolone | 521119 | 0.70 | 0.57 | 0.57 | 3 |
| Steroids | Estrone | 53167 | 3.6[b] | 0.097 | 0.11 | 2 |
| Steroids | Hydrocortisone | 50237 | 100 | 0.010 | 0.080 | 9 |
| Steroids | Progesterone | 57830 | 100 | 0.0020 | 0.0080 | 11 |
| Steroids | 4-Hydroxyandrostenedione | 566483 | 300 | 0.0017 | 0.014 | 3 |
| Steroids | Cortisone | 53065 | 2884 | 0.00076 | 0.0075 | 11 |
| Steroids | Trenbolone acetate | 10161349 | 1000 | 0.00030 | 0.00040 | 2 |
| Steroids | 4-Androstene-3,17-dione | 63058 | 2291 | 0.00011 | 0.00017 | 2 |
| Steroids | Medroxyprogesterone | 520854 | 5000 | 0.000030 | 0.00018 | 4 |

# Annex C

Supportive information to Chapter 6

# Annex C

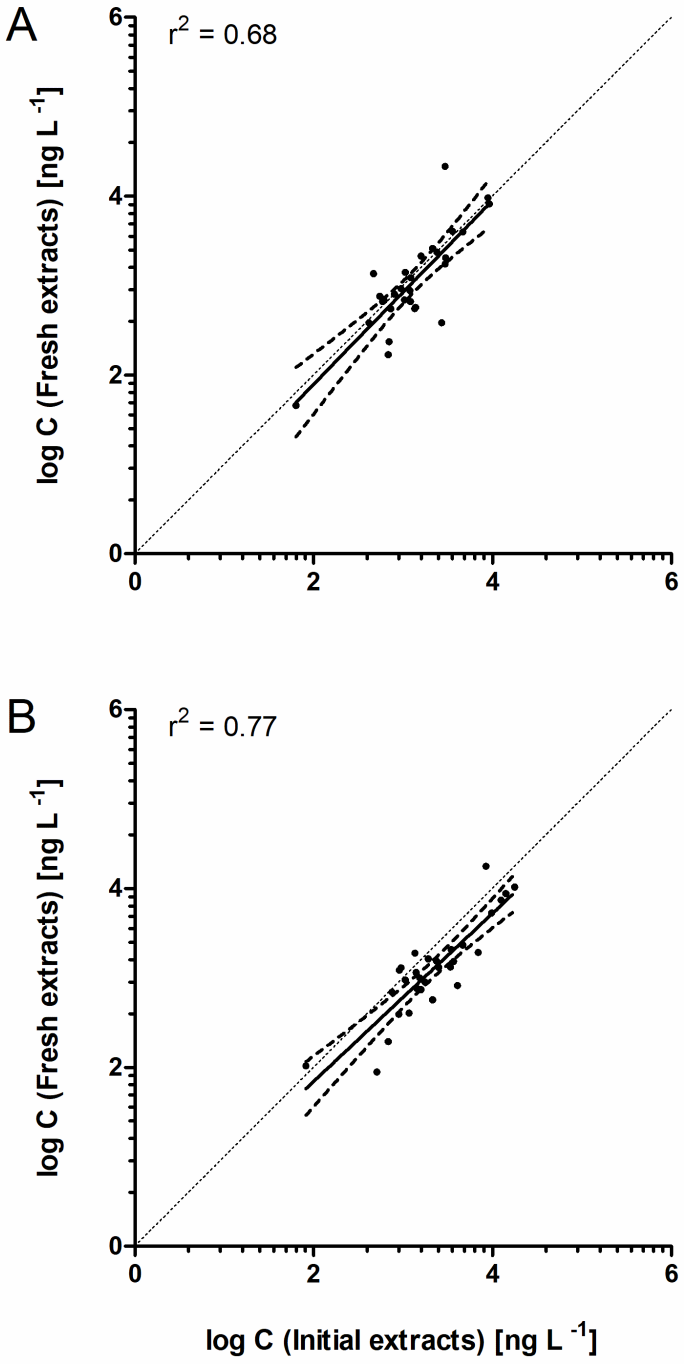


**Figure C1: Correlation plots of extracts Speedisk extraction 1 and 2 for harbor (A) and sea (B). Extracts 1 were extracted immediately after sampler recovery. Extracts 2 were extracted 16 months after sampler recovery. Shown are the log concentrations for target compounds quantified in both extracts (average of triplicates for extraction 1 and average of duplicates for extraction 2) in ng $L^{-1}$. The solid line is the best fit line, striped lines represent the 95% confidence intervals and the dotted line indicates the 1:1 identity line. $R^2$ denotes the correlation coefficient of the best fit line.**

**Table C1 Measured target compound concentrations (C) in the Speedisk extracts from the harbor of Zeebrugge and the coastal sampling location near Zeebrugge from sampling campaign 1. All values are indicated as average of triplicates (E1) or duplicates (E2) in ng $L^{-1}$. SD gives the respective standard deviation. E1 and E2 describe extraction 1 and 2, A1 and A2 stand for first and second analysis.**

| | HZ | | | | | | OZ_MOW1 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E1_A1 | | E1_A2 | | E2_A2 | | E1_A1 | | E1_A2 | | E2_A2 | |
| | C | SD | C | SD | C | SD | C | SD | C | SD | C | SD |
| Acyclovir | 2,700 | 2,200 | 480 | 90 | 390 | 52 | NA | NA | NA | NA | NA | NA |
| Alprazolam | NA | NA | NA | NA | NA | NA | 130 | NA | NA | NA | NA | NA |
| Amantadine | 3,600 | 87 | 1,300 | 78 | 4,100 | 1,100 | 12,000 | 1,200 | 4,700 | 690 | 7,400 | 920 |
| Atenolol | 3,000 | 64 | 910 | 10 | 22,000 | 9,400 | 8,500 | 3,800 | 2,700 | 1,100 | 18,000 | 9,200 |
| Atrazine | 1,200 | 40 | 230 | 57 | 660 | 46 | 2,500 | NA | 1,100 | 170 | 1,300 | 270 |
| Bezafibrate | 1,400 | 170 | 210 | 26 | 550 | 42 | 4,100 | 220 | 810 | 160 | 820 | 34 |
| Bisoprolol | 1,200 | 100 | 360 | 40 | 880 | 200 | 1,600 | 320 | 750 | 120 | 740 | 97 |
| Carbamazepine | 8,900 | 290 | 5,200 | 510 | 9,600 | 1,400 | 18,000 | 2,800 | 11,000 | 1,600 | 10,000 | 720 |
| Chloridazon | 2,400 | 110 | 1,600 | 58 | 2,400 | 380 | 1,400 | 2 | 1,600 | 380 | 1,200 | 150 |
| Ciprofloxacin | NA | NA | NA | NA | 270 | NA | NA | NA | NA | NA | NA | NA |
| Clarithromycin | 700 | 270 | 170 | 43 | 240 | 5 | 680 | NA | 180 | 50 | 200 | 28 |
| Clofibric acid | NA | NA | NA | NA | NA | NA | 240 | 6 | NA | NA | NA | NA |
| Clothianidin | 680 | 11 | 160 | 3 | 170 | 21 | 1,200 | 120 | 420 | 79 | 400 | 2 |
| Diazepam | 99 | 14 | NA | NA | NA | NA | 180 | NA | 89 | 21 | NA | NA |
| Dichlorophen-oxyacetic acid | 3,000 | 170 | 620 | 16 | 1,800 | 190 | 2,100 | 610 | 510 | 97 | 570 | 120 |
| Diclofenac | 1,600 | 510 | NA | NA | 2,200 | 290 | 4,700 | 2,600 | 820 | 110 | 2,300 | 1 |
| Dimethoate | 210 | 5 | 140 | 10 | NA | NA | NA | NA | 90 | NA | NA | NA |
| Dinoseb | NA | NA | NA | NA | 180 | NA | 300 | 52 | NA | NA | NA | NA |
| Diuron | 2,100 | 88 | 1,200 | 170 | 2,600 | 490 | 3,500 | NA | 2,300 | 240 | 2,100 | 250 |
| Flufenacet | 420 | 58 | 560 | 120 | 380 | 63 | 2,400 | NA | 1,900 | 190 | 1,500 | 490 |
| Flumequine | NA | NA | NA | NA | 100 | 21 | 82 | 3 | 110 | 21 | 100 | 18 |
| Gatifloxacin | NA | NA | 160 | 48 | NA | NA | NA | NA | 1,900 | 720 | 1,800 | 920 |
| Imidacloprid | 950 | 32 | 770 | 9 | 930 | 12 | 1,500 | 110 | 1,900 | 440 | 1000 | 15 |

| | HZ | | | | | | OZ_MOW1 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E1_A1 | | E1_A2 | | E2_A2 | | E1_A1 | | E1_A2 | | E2_A2 | |
| | C | SD | C | SD | C | SD | C | SD | C | SD | C | SD |
| Irgarol | 730 | 47 | 210 | 9 | 550 | 250 | 210 | NA | NA | NA | NA | NA |
| Isoproturon | 4,700 | 130 | 2,100 | 220 | 4,000 | 610 | 9,800 | NA | 6,600 | 1,300 | 5,300 | 820 |
| Lamivudine | NA | NA | NA | NA | NA | NA | 910 | 430 | 450 | 110 | 1,200 | 200 |
| Linuron | 280 | 17 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Mecoprop | 3,000 | 210 | 690 | 4 | 2,100 | 88 | 7,000 | 1,700 | 1,700 | 500 | 1,900 | 240 |
| Methylparaben | NA | NA | NA | NA | 1,200 | 370 | 2,300 | NA | NA | NA | NA | NA |
| Metolachlor | 1,400 | 210 | 500 | 140 | 570 | 170 | 3,400 | NA | 1,700 | 330 | 1,300 | 210 |
| Metoprolol | 1,000 | 83 | 250 | 21 | 690 | 200 | 3,700 | 550 | 1,300 | 320 | 1,500 | 120 |
| Metronidazole | 100 | 8 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Nevirapine | 190 | NA | 220 | 78 | NA | NA | 750 | 16 | 700 | 150 | 700 | 440 |
| Oseltamivir ethylester | NA | NA | NA | NA | 2,100 | 2,200 | NA | NA | NA | NA | 1,000 | 380 |
| Paracetamol | 1,500 | 290 | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Paroxetine | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | 640 | 410 |
| Pirimicarb | 200 | 11 | 46 | 6 | NA | NA | 510 | 55 | 180 | 31 | 89 | 45 |
| Propranolol | 470 | 102 | 290 | 21 | 1,400 | 380 | 1,400 | 170 | 790 | 87 | 760 | 21 |
| Propylparaben | NA | NA | NA | NA | 2,400 | 66 | NA | NA | NA | NA | 1,300 | 7 |
| Salicylic acid | 1,400 | NA | NA | NA | NA | NA | 1,400 | 380 | NA | NA | NA | NA |
| Simazine | 620 | 74 | 310 | 34 | 680 | 27 | 1,100 | 47 | 730 | 98 | 950 | 33 |
| Sotalol | 9,200 | 210 | 3,300 | 71 | 8,200 | 1,200 | 14,000 | 3,000 | 6,900 | 2,100 | 8,800 | 210 |
| Sulfamethazine | 64 | 19 | 34 | 1 | 46 | NA | 140 | 33 | 64 | 9 | NA | NA |
| Sulfamethoxa-zole | 1,100 | 65 | 440 | 46 | 1,400 | 12 | 1,400 | 170 | 1,100 | 280 | 1,900 | 210 |
| Terbuthylazine | 1,200 | 82 | 640 | 140 | 1,200 | 52 | 1,900 | NA | 1,200 | 100 | 1,600 | 250 |
| Terbutryn | NA | NA | NA | NA | NA | NA | 760 | NA | NA | NA | NA | NA |
| Thiacloprid | 800 | 60 | 600 | 120 | 810 | 120 | 1,800 | 1 | 1,700 | 250 | 910 | 180 |
| Thiamethoxam | 550 | 120 | 600 | 64 | 760 | 50 | 950 | 120 | 1,400 | 320 | 1,300 | 4 |
| Trimethoprim | 590 | 48 | 140 | 6 | 660 | 19 | 900 | 64 | 280 | 57 | 390 | 1 |
| Venlafaxine | 2,700 | 220 | NA | NA | NA | NA | 6,400 | 81 | 110 | NA | NA | NA |

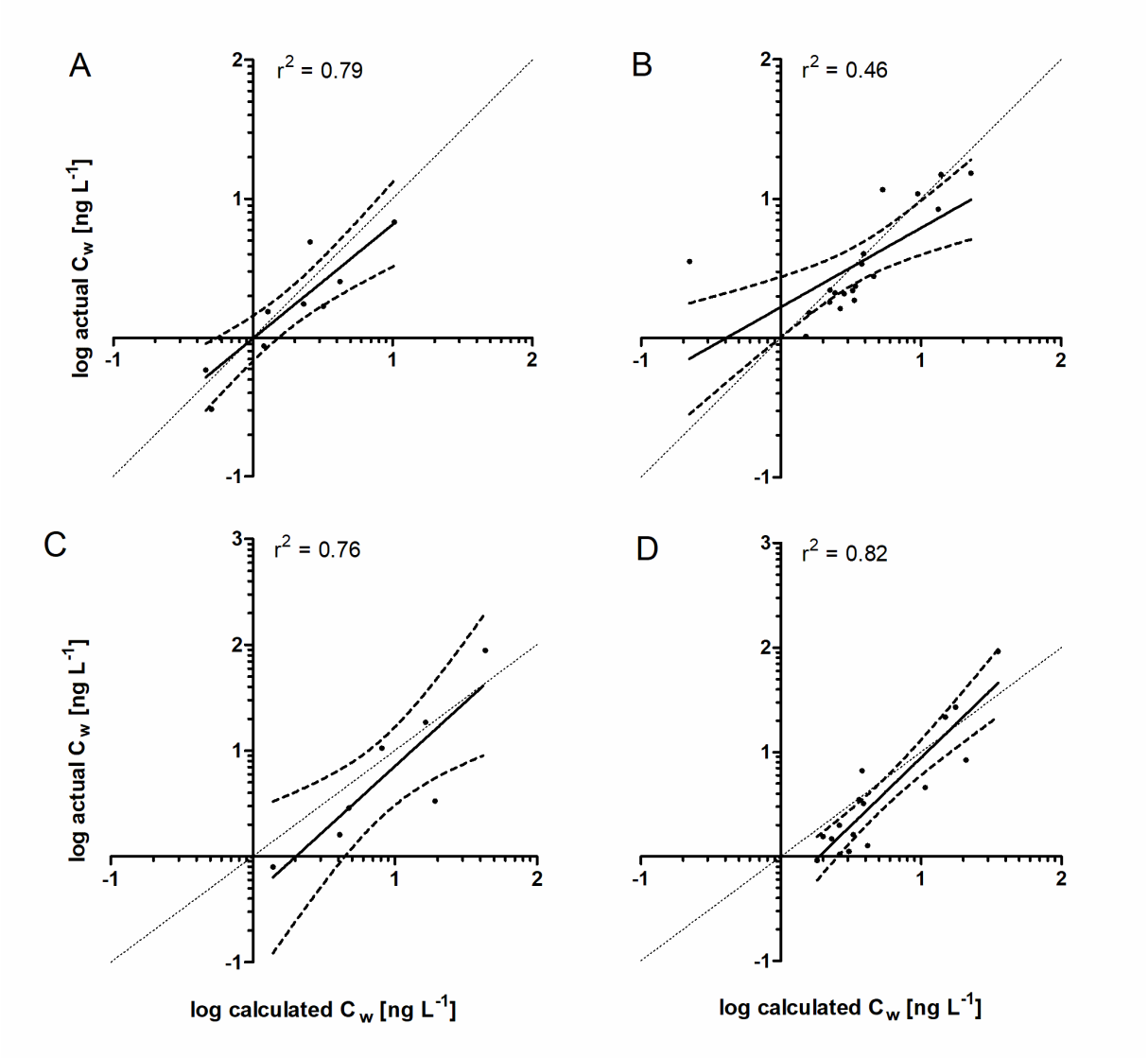


**Figure C2: Correlation plots comparing the calculated and actual test medium concentrations ($C_w$) of the target compound in the Speedisk extracts. Shown are the $C_w$ of test 2 for harbor (A) and sea (B) and test 3 for harbor (C) and sea (D). The solid line is the best fit line, striped lines represent the 95% confidence intervals and the dotted line indicates the 1:1 identity line. $R^2$ denotes the correlation coefficient of the best fit line.**

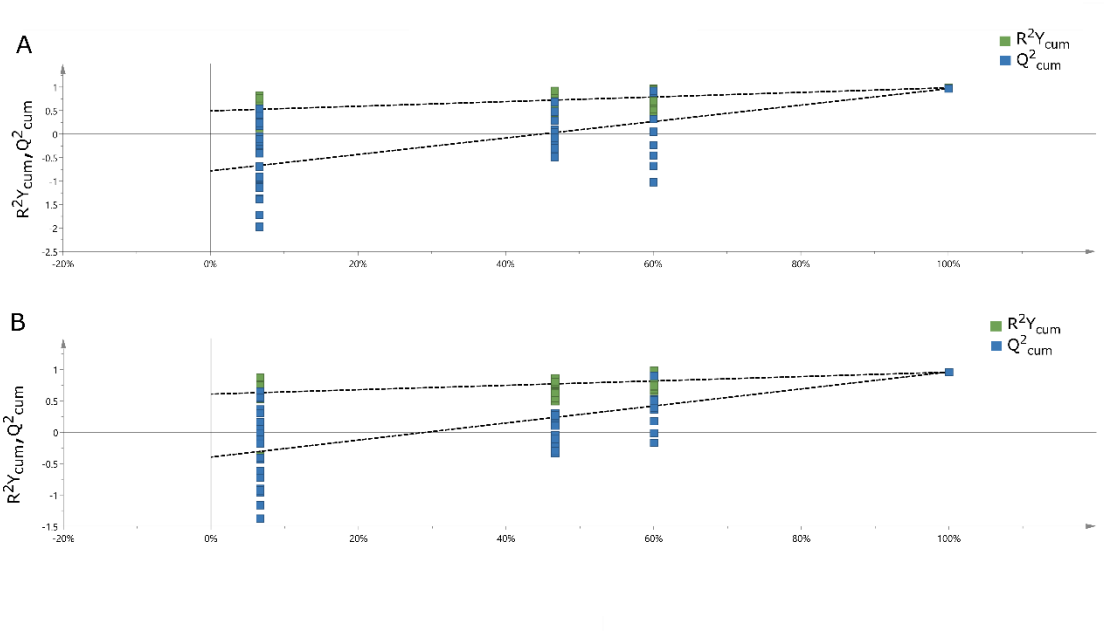


**Figure C3: Permutation (100 times Monte Carlo simulation) testing of OPLS-DA models for harbor (A) and sea (B).**

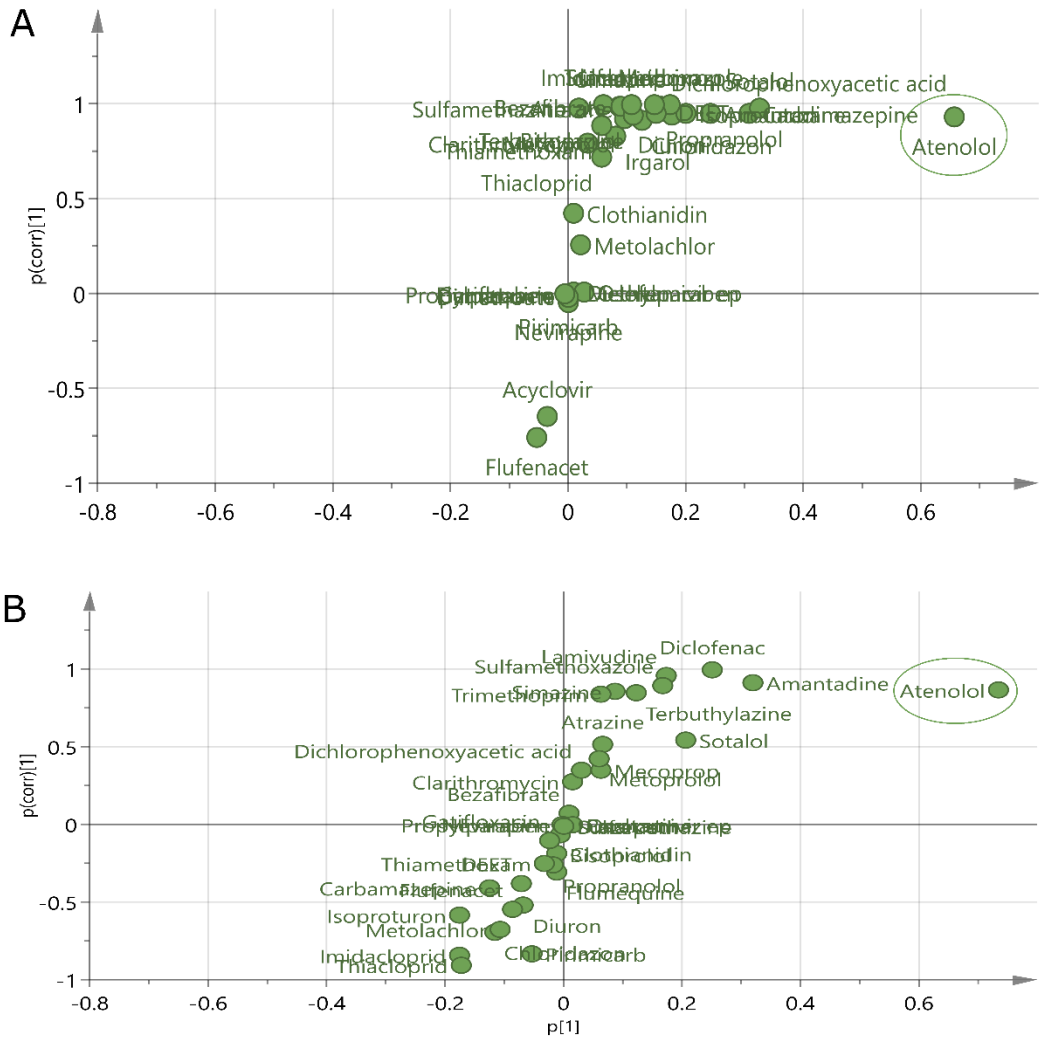


**Figure C4: S-plots for harbor (A, $R^2X = 0.886$) and sea (B, $R^2X = 0.846$) combining the contribution/covariance (p[1]) and reliability/correlation (p(corr)[1]) of the OPLS-DA models.**

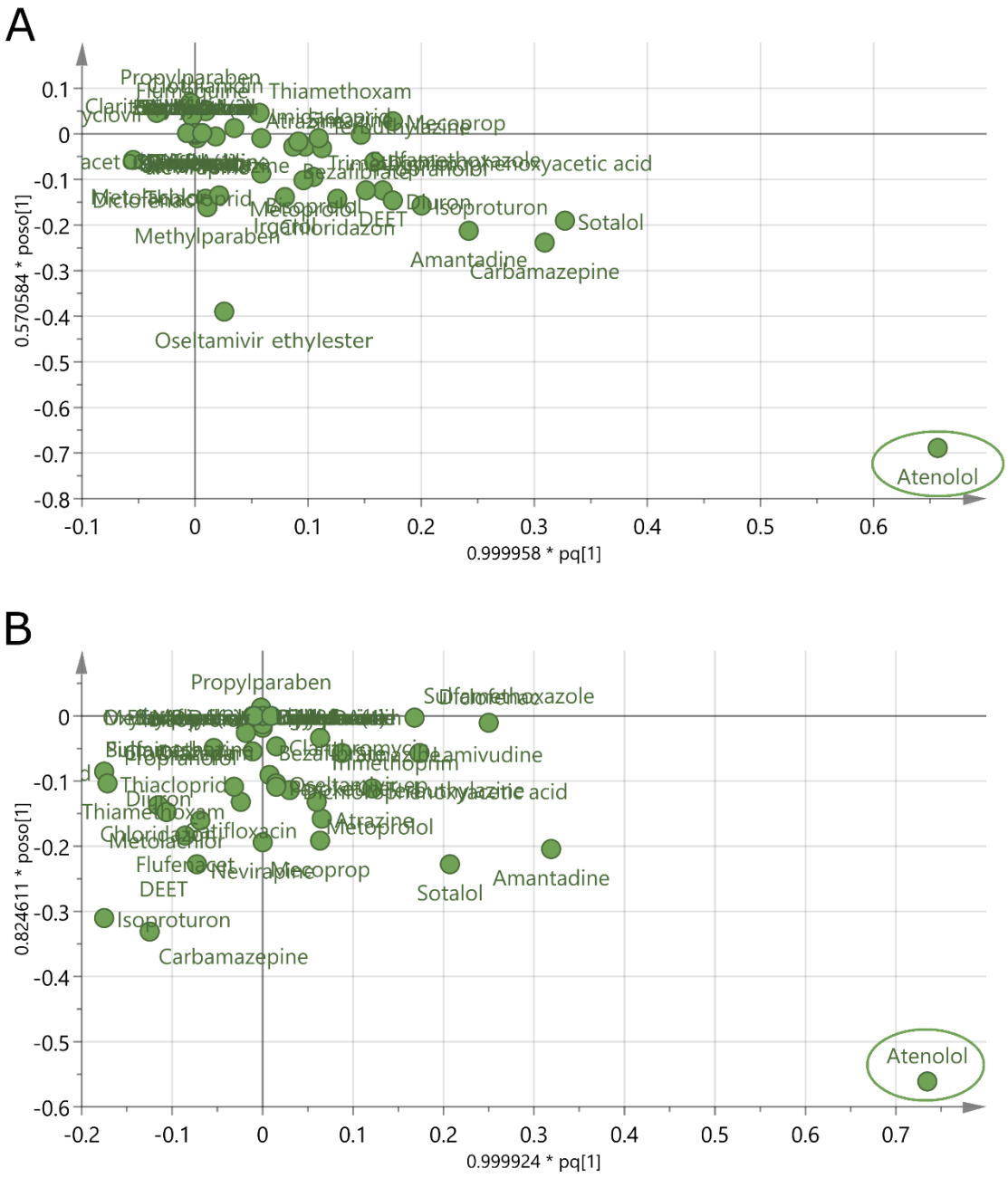


**Figure C5: OPLS-DA compound loading plots for harbor (A, $R^2X = 0.886$) and sea (B, $R^2X = 0.846$) models.**

**Table C2 Growth inhibition [%] of atenolol tested individually. Log C indicates the logarithmic concentration in ng $L^{-1}$, μ describes the mean growth rate [$d^{-1}$] and $I_\mu$ the calculated growth rate in %. SD indicates the respective standard deviation. CTL represents the control treatments.**

| Log C [ng $L^{-1}$] | μ ($d^{-1}$) | SD ($d^{-1}$) | $I_\mu$ [%] | SD [%] |
|---|---|---|---|---|
| 2.0 | 1.2 | 0.008 | -0.06 | 0.69 |
| 1.7 | 1.2 | 0.004 | -0.74 | 0.34 |
| 1.4 | 1.1 | 0.066 | 4.6 | 5.5 |
| 1.1 | 1.2 | 0.005 | 1.7 | 0.44 |
| 0.80 | 1.2 | 0.003 | 1.8 | 0.29 |
| 0.49 | 1.1 | 0.033 | 4.3 | 2.7 |
| 0.19 | 1.1 | 0.057 | 5.7 | 4.8 |
| -0.11 | 1.2 | 0.007 | 1.9 | 0.61 |
| -0.41 | 1.2 | 0.015 | 2.3 | 1.3 |
| -0.71 | 1.2 | 0.006 | 1.5 | 0.49 |
| -1.0 | 1.2 | 0.007 | 0.15 | 0.58 |
| -1.3 | 1.2 | 0.023 | -0.11 | 1.9 |
| -1.6 | 1.2 | 0.012 | 1.7 | 1.0 |
| -1.9 | 1.2 | 0.018 | 2.0 | 1.5 |
| -2.2 | 1.2 | 0.007 | 3.1 | 0.61 |
| -2.5 | 1.2 | 0.006 | 3.7 | 0.54 |
| -2.8 | 1.2 | 0.005 | 2.3 | 0.41 |
| -3.1 | 1.2 | 0.008 | 2.6 | 0.68 |
| 0.0 (CTL) | 1.2 | 0.011 | -0.063 | 0.69 |

# Annex D

Supportive information to Chapter 7

# Annex D

**Table D1 Measured concentrations in grab water samples from sampling campaigns 2,3 and 5. Given is the average concentration (mean) and the standard deviation (SD) from samples collected at sampler deployment and retrieval. All concentrations are provided in ng $L^{-1}$.**

| Compounds | SC2 HO | | SC2 HZ | | SC2 OZ_MOW1 | | SC3 HO | | SC3 HZ | | SC3 OO_X | | SC5 HO | | SC5 HZ | | MDL[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | |
| Acetamiprid | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 0.7 | 0.2 | [a] | [a] | 0. 3 |
| Alachlor | [a] | [a] | 45 | 3 | 46 | 8 | [a] | [a] | [a] | [a] | [a] | [a] | 19 | 5 | [a] | [a] | 8.3 |
| Amantadine | 4.2 | 0.7 | 7.5 | 0.9 | 5.7 | 6.7 | 3.7 | 2.7 | 4.8 | 0.6 | 1.9 | 1.8 | 4.7 | 0.6 | 4 | 0.3 | 0.08 |
| Atenolol | 58 | 25 | 20 | 20 | 4.8 | 0.6 | 18 | 11 | 5 | 1.6 | 1.7 | 1.5 | 27 | 18 | 4.1 | 2.5 | 0.07 |
| Atrazine | 3.6 | 0.9 | 1.8 | 0.5 | 1.2 | 0.4 | 2.5 | 0.3 | 1.5 | 0.4 | 0.9 | 0.4 | 3.5 | 0.7 | 2 | 0.7 | 0.4 |
| Bezafibrate | 6.8 | 1.1 | 3.1 | 2.8 | 1.9 | 0.3 | 3 | 1.6 | 4.8 | 5.6 | 0.9 | 0.3 | 5 | 3 | 1.2 | 0.8 | 0.4 |
| Bisoprolol | 23 | 7 | 6.2 | 3.3 | 1.5 | 1.2 | 16 | 8 | 2.3 | 0.2 | 0.5 | 0.2 | 29 | 8 | 3.8 | 2.6 | 0.05 |
| Butylparaben | [a] | [a] | [a] | [a] | [a] | [a] | 8.1 | 0.5 | 1.4 | NA | [a] | [a] | 0.9 | 0.1 | [a] | [a] | 0.5 |
| Carbamazepine | 31 | 16 | 13 | 1 | 7.2 | 7 | 29 | 7 | 11 | 3 | 5.5 | 4 | 32 | 4 | 9.8 | 1.6 | 1.9 |
| Chloridazon | 3.9 | 2.1 | 4.1 | 0.6 | 2.4 | 1.7 | 5 | 3.2 | 2.8 | 0.2 | 2.3 | 0.9 | 22 | 27 | 3.2 | 2.4 | 0.08 |
| Chloroxylenol | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 680 | 80 | [a] | [a] | [a] | [a] | 487 |
| Clarithromycin | 6.4 | 3.4 | 2.3 | NA | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 3.9 | 0.7 | [a] | [a] | 1.9 |
| Clothianidin | 3.5 | 2.6 | 1.4 | 1.1 | 0.7 | 0.1 | 3.1 | 0.3 | 0.3 | NA | 0.2 | NA | 0.9 | 0.4 | 0.4 | 0.2 | 0.2 |
| DEET | 13 | 4 | 7 | 2.8 | 6.6 | 4.7 | 7.1 | 0.1 | 5.2 | 2.1 | 4 | 0.1 | 11 | 1 | 5.2 | 0.4 | 1.1 |
| Diazepam | 0.1 | NA | [a] | [a] | [a] | [a] | [a] | [a] | 0.1 | NA | [a] | [a] | 0.2 | 0.02 | [a] | [a] | 0.06 |
| Dichlorophenoxyacetic acid | 8.4 | 2.6 | 5.6 | [a] | [a] | [a] | 7.7 | 4.4 | 1.4 | 0.7 | 0.9 | [a] | 8.5 | 4.9 | 1.8 | 0.7 | 0.8 |
| Diclofenac | 85 | 23 | 27 | 21 | 9.8 | 1 | 21 | 14 | [a] | [a] | 24 | 17 | 43 | 22 | 11 | 1 | 0.4 |
| Dimethoate | [a] | [a] | [a] | [a] | [a] | [a] | 0.8 | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 0.3 |
| Dinoseb | [a] | [a] | [a] | [a] | [a] | [a] | 7.3 | 1.8 | 2.2 | 1.6 | [a] | [a] | [a] | [a] | [a] | [a] | 5.1 |
| Diuron | 12 | 6 | 5.7 | 0.7 | 2.9 | [a] | 4 | 1.2 | 2.4 | 0.05 | 1.8 | [a] | 6.8 | 0.3 | 3.7 | 1.9 | 1.5 |
| Efavirenz | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 1.4 | 0.3 | [a] | [a] | [a] | [a] | [a] | [a] | 0.2 |
| Flufenacet | 21 | 22 | 9.4 | 11 | 1.9 | 0.2 | 1.3 | 0.2 | 1.1 | 0.2 | [a] | [a] | 3.3 | 2.1 | 1.8 | 0.6 | 0.7 |

| Compounds | SC2 HO | | SC2 HZ | | SC2 OZ_MOW1 | | SC3 HO | | SC3 HZ | | SC3 OO_X | | SC5 HO | | SC5 HZ | | MDL[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | |
| Flumequine | [a] | [a] | 1.6 | 0.1 | 2.1 | 0.1 | [a] | [a] | 12 | 6 | [a] | [a] | [a] | [a] | [a] | [a] | 3.4 |
| Gatifloxacin | 1.1 | 0.1 | 3.5 | NA | 5.2 | 0.1 | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 6.9 |
| Ifosfamide | [a] | [a] | [a] | [a] | [a] | [a] | 0.3 | 0.1 | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 0.05 |
| Imidacloprid | 7.5 | 2.1 | 6 | 6.1 | 1 | 0.1 | 6.1 | 5.1 | 0.8 | 0.6 | 0.6 | 0.1 | 3.1 | 1.5 | 0.9 | 0.7 | 0.3 |
| Irgarol | [a] | [a] | 0.3 | 0.1 | 0.3 | [a] | 0.6 | 0.5 | 0.4 | 0.2 | 0.2 | 0.02 | 0.2 | 0.1 | 1 | | 0.1 |
| Isoproturon | 43 | 37 | 26 | 31 | 9.4 | 7.8 | 4.2 | 3.7 | 2.1 | 1 | 1.1 | 1.3 | 4.7 | 0.5 | 1.4 | 0.04 | 0.09 |
| Ketoprofen | [a] | [a] | [a] | [a] | [a] | [a] | 0.8 | 0.3 | [a] | [a] | [a] | [a] | 18 | 22 | 41 | 4 | 0.2 |
| Linuron | 4.4 | 3 | 0.7 | 0.1 | 0.8 | 0.1 | 3.6 | 3.4 | 0.9 | 0.7 | 0.5 | NA | 2.9 | 2.1 | 0.5 | NA | 0.4 |
| Mecoprop | 8.5 | 4.1 | 4 | 2.8 | 2.7 | [a] | 8.5 | 1.3 | 2.5 | 0.7 | 1.2 | 0.4 | 8.5 | 1.1 | 2.1 | 0.1 | 0.3 |
| Methylparaben | 5.3 | 1.9 | [a] | [a] | 7 | 3.6 | 2.8 | 0.5 | [a] | [a] | [a] | [a] | 2.7 | 0.2 | 2.6 | 0.3 | 2.3 |
| Metolachlor | 8.4 | 6.6 | 3.1 | 0.2 | 3.6 | 0.5 | 2.8 | 1.2 | 1.9 | 0.5 | 0.7 | 0.02 | 5.6 | 3.3 | 1.6 | 0.3 | 0.3 |
| Metoprolol | 11 | 7 | 4.7 | 1.6 | 3.1 | 3.8 | 6.6 | 0.9 | 3.4 | 0.4 | 0.6 | 0.2 | 7.2 | 0.7 | 1.8 | 1.3 | 0.1 |
| Metronidazole | 2.8 | 0.3 | [a] | [a] | [a] | [a] | 0.5 | 0.3 | 0.3 | 0.1 | [a] | [a] | 1.6 | 1.6 | 0.3 | NA | 0.2 |
| Nalidixic acid | 6.2 | 0.5 | 6.4 | 0.2 | 9.6 | 3 | [a] | [a] | [a] | [a] | 3.2 | 0.4 | [a] | [a] | [a] | [a] | 0.7 |
| Naproxen | 57 | 5 | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 41 | 3 | [a] | [a] | 11 |
| Nevirapine | 0.6 | 0.1 | 0.4 | NA | 0.4 | 0.1 | 0.7 | 0.1 | 0.3 | 0.1 | [a] | [a] | 1 | 0.3 | 0.5 | 0.1 | 0.2 |
| Oxybenzone | 7.5 | 0.5 | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 5.8 |
| Paracetamol | 30 | 13 | 16 | 5 | 18 | 7 | 4.1 | 0.2 | 4.8 | [a] | 12 | 9 | 18 | 18 | 7.1 | 6 | 0.9 |
| Pentachlorophenol | [a] | [a] | [a] | [a] | [a] | [a] | 11 | 2 | [a] | [a] | [a] | [a] | 11 | 1 | 12 | 1 | 8.9 |
| Piperonylbutoxide | 13 | 1 | 1.1 | NA | [a] | [a] | 2.4 | 0.3 | 2.5 | 1.5 | [a] | [a] | 5 | 2.4 | 1.2 | 0.2 | 0.8 |
| Pirimicarb | 2 | 0.6 | 0.8 | 0.9 | 0.2 | 0.01 | 0.4 | 0.4 | 0.1 | NA | [a] | [a] | 1.4 | 0.1 | 0.2 | NA | 0.08 |
| Propranolol | 9 | 2.7 | 2.7 | 1.8 | 0.6 | 0.3 | 4.5 | 2.1 | 0.7 | 0.2 | 0.5 | NA | 7.1 | 1.8 | 1.4 | 0.8 | 0.3 |
| Propylparaben | 1.5 | 0.1 | [a] | [a] | [a] | [a] | [a] | [a] | 0.9 | 0.3 | 0.5 | NA | 1.2 | 0.1 | 1.1 | 0.3 | 0.2 |
| Rimantadine | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 0.2 | NA | [a] | [a] | [a] | [a] | [a] | [a] | 0.09 |
| Simazine | 1.3 | 0.4 | 0.9 | 0.2 | 0.7 | 0.1 | 1 | 0.04 | 0.6 | 0.02 | 0.3 | 0.1 | 3.1 | 1.5 | 0.8 | 0.1 | 0.02 |
| Sotalol | 153 | 4 | 45 | 31 | 12 | 7 | 70 | 45 | 14 | 7 | 4.9 | 4.8 | 103 | 22 | 15 | 9 | 0.08 |
| Sulfadoxin | 0.6 | NA | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 0.4 | 0.3 | [a] | [a] | 0.1 |
| Sulfamethazine | 1.4 | 0.1 | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 1.1 | 1.2 | 0.3 | NA | 0.2 |

| Compounds | SC2 HO | | SC2 HZ | | SC2 OZ_MOW1 | | SC3 HO | | SC3 HZ | | SC3 OO_X | | SC5 HO | | SC5 HZ | | MDL[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | mean | SD | |
| Sulfamethoxazole | 3.6 | 3.5 | 3.5 | 2.2 | 4.4 | 4.7 | 11 | 3 | 3.6 | 2.2 | 2.7 | 2.5 | 15 | 11 | 4.8 | 3.3 | 0.5 |
| Terbuthylazine | 12 | 9 | 7.1 | 3 | 3.4 | 2.7 | 2.8 | 0.01 | 2.5 | 0.6 | 1.2 | 0.6 | 9.9 | 5.1 | 1.7 | 0.2 | 0.1 |
| Terbutryn | 1.4 | 0.1 | [a] | [a] | [a] | [a] | 1.1 | 0.1 | [a] | [a] | 1.4 | 0.3 | 1.3 | 0.2 | [a] | [a] | 0.5 |
| Thiacloprid | 33 | 44 | 1.8 | 0.4 | 0.4 | 0.1 | 2.9 | 1.4 | 0.9 | 0.7 | 0.5 | 0.6 | 10 | 11 | 0.9 | 0.9 | 0.07 |
| Thiamethoxam | 7.5 | 4.9 | 1.7 | 0.1 | 2.1 | NA | 54 | 9 | 1.4 | 0.3 | 1.6 | 0.4 | 7 | 4.4 | 2 | 0.1 | 1 |
| Trimethoprim | 4.6 | 3 | 1 | 0.8 | 0.4 | 0.2 | 1.9 | 0.7 | 0.5 | 0.1 | 0.2 | 0.1 | 1.8 | 1.1 | 0.4 | 0.2 | 0.03 |
| Venlafaxine | 16 | 5 | 6.7 | 1.5 | 3 | 2.5 | 17 | 1 | 6 | 1.1 | 1.8 | 0.9 | 22 | 6 | 5.6 | 0.4 | 1 |

[a] < MDL (Method detection limit)

[b] [20]

NA: Not applicable, no SD since substance only measured in one replicate.

**Table D2 Overview of the calculated relative enrichment factors ($REF_i$) for all substances across all sampling locations and campaigns.**

| Compound | SC2 HO | SC2 HZ | SC2 OZ_MOW1 | SC3 HO | SC3 HZ | SC3 OO_X | SC5 HO | SC5 HZ |
|---|---|---|---|---|---|---|---|---|
| Amantadine | 30 | 30 | 50 | 32 | 24 | 51 | 21 | 36 |
| Atenolol | 3 | 4 | 23 | [a] | [a] | [a] | [a] | [a] |
| Atrazine | 18 | 42 | 115 | [a] | [a] | [a] | 22 | 25 |
| Bezafibrate | 21 | 76 | 405 | 73 | 42 | 492 | [a] | [a] |
| Bisoprolol | 6 | 10 | 19 | 8 | 41 | [a] | 11 | 11 |
| Carbamazepine | 14 | 31 | 89 | [a] | [a] | [a] | 18 | 31 |
| Chloridazon | 21 | 21 | 56 | 14 | 29 | 33 | 10 | 21 |
| Clarithromycin | 3 | 5 | [a] | [a] | [a] | [a] | 4 | [a] |
| Clothianidin | 2 | 5 | 8 | 1 | 17 | 17 | 7 | 13 |
| DEET | 15 | 37 | 16 | [a] | [a] | [a] | 14 | 21 |
| Dichlorophenoxyacetic acid | 4 | 18 | | [a] | [a] | [a] | 16 | 32 |
| Diclofenac | 6 | 9 | 20 | 3 | [a] | 40 | 15 | 10 |
| Diuron | 5 | 11 | 23 | 11 | 19 | 24 | 8 | 10 |

| Compound | SC2 HO | SC2 HZ | SC2 OZ_MOW1 | SC3 HO | SC3 HZ | SC3 OO_X | SC5 HO | SC5 HZ |
|---|---|---|---|---|---|---|---|---|
| Flufenacet | 6 | 8 | 24 | 15 | 11 | [a] | 24 | 16 |
| Flumequine | [a] | [a] | [a] | [a] | 1 | [a] | [a] | [a] |
| Imidacloprid | 19 | 11 | 78 | 7 | 71 | 42 | 31 | 46 |
| Irgarol | | 46 | 35 | 28 | 30 | 50 | 18 | 5 |
| Isoproturon | 8 | 5 | 23 | [a] | [a] | [a] | 16 | 29 |
| Ketoprofen | [a] | [a] | [a] | [a] | [a] | [a] | 4 | [a] |
| Linuron | 15 | 107 | 136 | 21 | 70 | 108 | 12 | [a] |
| Mecoprop | 6 | 12 | 20 | 6 | 23 | 43 | 8 | 27 |
| Metolachlor | 12 | 42 | 64 | 17 | 21 | 113 | 13 | 28 |
| Metoprolol | 5 | 13 | 27 | [a] | [a] | [a] | 14 | 19 |
| Metronidazole | 9 | [a] | [a] | [a] | [a] | [a] | 10 | 17 |
| Naproxen | [a] | [a] | [a] | [a] | [a] | [a] | 27 | [a] |
| Nevirapine | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 49 |
| Oxybenzone | 14 | [a] | [a] | [a] | [a] | [a] | [a] | [a] |
| Paracetamol | 2 | 1 | 1 | [a] | [a] | [a] | [a] | [a] |
| Piperonylbutoxide | 3 | 18 | [a] | [a] | [a] | [a] | 13 | [a] |
| Pirimicarb | 40 | 259 | 237 | [a] | [a] | [a] | 10 | 17 |
| Propranolol | 4 | 8 | 21 | 7 | 51 | 16 | 12 | 20 |
| Simazine | 31 | 49 | 80 | [a] | [a] | [a] | [a] | [a] |
| Sotalol | 5 | 8 | 43 | 7 | 39 | 49 | 15 | 16 |
| Sulfadoxin | 2 | [a] | [a] | [a] | [a] | [a] | 5 | [a] |
| Sulfamethazine | 5 | [a] | [a] | [a] | [a] | [a] | 6 | 7 |
| Sulfamethoxazole | 37 | 33 | 38 | 10 | 34 | 21 | 13 | 19 |
| Terbuthylazine | 16 | 37 | [a] | [a] | [a] | [a] | 16 | 27 |
| Thiacloprid | 1 | 23 | 23 | [a] | [a] | [a] | 7 | 15 |
| Thiamethoxam | 10 | 22 | 23 | 1 | 52 | 19 | 30 | 26 |
| Trimethoprim | 5 | 8 | 14 | [a] | [a] | [a] | 34 | 37 |
| Venlafaxine | 4 | 8 | 13 | 8 | 20 | 40 | 9 | 12 |
| **$REF_{geomean}$** | **8** | **17** | **33** | **9** | **25** | **44** | **13** | **13** |

[a] No $REF_i$ defined due to either no detection in speedisk extract or grab water sample.

**Table D3 Calibration series used for the conversion of fluorescence measurements to cell counts for biotesting of the enriched speedisk extracts of the three sampling campaigns 2,3 and 5 (SC2, SC3 and SC5).**

| Cell count [cells $mL^{-1}$] | Fluorescence SC2 | Fluorescence SC3 | Fluorescence SC5 |
|---|---|---|---|
| 10000 | 2149 | 2031 | 2021 |
| 25000 | 3424 | 3761 | 5908 |
| 50000 | 5358 | 6663 | 10945 |
| 75000 | 8757 | 9372 | 29684 |
| 100000 | 11217 | 12370 | 3513 |
| 200000 | 20565 | 23143 | 8451 |
| 300000 | 28933 | 32796 | 20297 |
| 400000 | 37914 | 42977 | 39147 |

**Table D4 Measured concentrations in (undiluted) speedisk $H_2O$ extracts from sampling campaign 2. Speedisks were deployed in triplicates (1-3) at three sampling locations: the harbor of Ostend (HO), the harbor of Zeebrugge (HZ) and a coastal sampling location near Zeebrugge (OZ_MOW1). MDL indicates the method detection limit. All concentrations are indicated in µg $L^{-1}$.**

| Compound | HO.1 | HO.2 | HO.3 | HZ.1 | HZ.2 | HZ.3 | OZ_MOW1.1 | OZ_MOW1.2 | OZ_MOW1.3 |
|---|---|---|---|---|---|---|---|---|---|
| Alprazolam | 0.13 | 0.091 | 0.11 | 0.073 | 0.088 | 0.074 | [a] | 0.16 | 0.092 |
| Amantadine | 2.5 | 2.1 | 3.0 | 4.4 | 4.7 | 4.4 | [a] | 5.6 | 5.8 |
| Atenolol | 3.0 | 2.6 | 3.9 | 1.3 | 1.4 | 1.5 | 2.1 | 2.3 | 2.1 |
| Atrazine | 1.3 | 1.3 | 1.4 | 1.6 | 1.6 | 1.4 | [a] | 2.7 | [a] |
| Bezafibrate | 2.9 | 2.8 | 3.0 | 4.7 | 4.6 | 4.9 | 16.1 | 14.0 | 15.9 |
| Bisoprolol | 2.5 | 2.3 | 3.2 | 1.2 | 1.3 | 1.3 | [a] | 0.85 | 0.27 |
| Butylparaben | [a] | [a] | [a] | 0.80 | 0.78 | [a] | [a] | [a] | [a] |
| Carbamazepine | 8.2 | 7.7 | 9.2 | 7.8 | 8.4 | 8.3 | 11 | 14 | 14 |
| Chloridazon | 1.4 | 1.4 | 2.0 | 1.6 | 1.7 | 1.7 | [a] | 3.1 | 2.4 |
| Clarithromycin | 0.34 | 0.34 | 0.42 | 0.22 | 0.26 | 0.19 | [a] | 0.080 | 0.075 |
| Clothianidin | 0.16 | 0.16 | 0.15 | 0.13 | 0.15 | 0.13 | 0.13 | 0.10 | 0.12 |

| Compound | HO.1 | HO.2 | HO.3 | HZ.1 | HZ.2 | HZ.3 | OZ_MOW1.1 | OZ_MOW1.2 | OZ_MOW1.3 |
|---|---|---|---|---|---|---|---|---|---|
| DEET | 4.1 | 3.8 | 4.2 | 5.0 | 6.0 | 4.5 | a | 3.6 | 0.67 |
| Dichlorophenoxyacetic acid | 0.68 | 0.62 | 0.66 | 1.4 | 3.6 | 0.88 | 0.44 | 0.38 | 0.41 |
| Diclofenac | 10 | 9.0 | 12 | 4.6 | 4.8 | 5.0 | 4.1 | 3.7 | 3.7 |
| Dimethoate | 0.10 | 0.077 | 0.12 | a | a | a | a | a | a |
| Dinoseb | a | 0.28 | 0.22 | 0.45 | 0.32 | a | a | a | a |
| Diuron | 1.2 | 1.1 | 1.2 | 1.2 | 1.3 | 1.2 | 1.5 | 1.2 | 1.4 |
| Ethylparaben | a | 0.25 | a | 0.25 | a | a | a | a | a |
| Flufenacet | 2.4 | 2.4 | 3.0 | 1.5 | 1.5 | 1.5 | a | 1.2 | 0.52 |
| Imidacloprid | 2.8 | 2.5 | 3.1 | 1.3 | 1.4 | 1.3 | a | 1.7 | 1.4 |
| Irgarol | 0.093 | 0.13 | 0.11 | 0.40 | 0.31 | 0.21 | a | 0.22 | 0.15 |
| Isoproturon | 6.7 | 5.8 | 7.1 | 2.5 | 2.9 | 2.5 | a | 4.0 | 4.8 |
| Ketoprofen | 0.94 | 0.95 | 1.1 | a | a | a | a | a | a |
| Lamivudine | a | a | a | a | 0.22 | a | a | a | a |
| Linuron | 1.3 | 1.2 | 1.4 | 1.7 | 1.4 | 1.4 | a | 2.2 | a |
| Mecoprop | 1.1 | 0.98 | 1.1 | 0.99 | 0.86 | 0.98 | 1.1 | 1.1 | 1.2 |
| Methylparaben | 0.47 | a | 0.30 | 0.44 | a | 0.31 | a | a | a |
| Metolachlor | 2.0 | 2.0 | 2.2 | 2.7 | 2.6 | 2.5 | a | 4.6 | a |
| Metoprolol | 1.2 | 1.1 | 1.4 | 1.1 | 1.2 | 1.2 | a | 2.1 | 1.2 |
| Metronidazole | 0.58 | 0.48 | 0.50 | 0.15 | 0.16 | 0.17 | a | a | a |
| Oxybenzone | a | a | 2.1 | a | a | a | a | a | a |
| Paracetamol | 0.96 | 0.97 | 1.6 | 0.41 | 0.36 | 0.36 | 0.38 | 0.27 | 0.35 |
| Piperonylbutoxide | 0.83 | 0.69 | 0.95 | 0.40 | 0.41 | 0.41 | a | a | a |
| Pirimicarb | 0.89 | 1.9 | 1.9 | 4.6 | 6.7 | 1.9 | a | 0.35 | 1.8 |
| Propranolol | 0.72 | 0.64 | 0.83 | 0.41 | 0.44 | 0.42 | a | 0.36 | 0.19 |
| Simazine | 0.80 | 0.79 | 0.81 | 0.85 | 0.84 | 0.88 | a | 1.43 | 0.81 |
| Sotalol | 13 | 12 | 17 | 7.0 | 7.3 | 7.5 | 10 | 10 | 10 |
| Sulfadoxin | 0.026 | 0.022 | 0.027 | a | a | a | a | a | a |
| Sulfamethazine | 0.13 | 0.11 | 0.15 | a | a | 0.049 | a | 0.054 | 0.029 |

| Compound | HO.1 | HO.2 | HO.3 | HZ.1 | HZ.2 | HZ.3 | OZ_MOW1.1 | OZ_MOW1.2 | OZ_MOW1.3 |
|---|---|---|---|---|---|---|---|---|---|
| Sulfamethoxazole | 2.6 | 2.5 | 2.9 | 2.3 | 2.3 | 2.3 | [a] | 3.7 | 3.0 |
| Terbuthylazine | 4.0 | 3.7 | 4.3 | 5.3 | 5.2 | 5.2 | [a] | [a] | [a] |
| Terbutryn | [a] | [a] | [a] | [a] | [a] | [a] | [a] | 0.68 | [a] |
| Thiacloprid | 0.85 | 0.77 | 1.11 | 0.80 | 0.84 | 0.78 | [a] | 0.20 | 0.18 |
| Thiamethoxam | 1.4 | 1.4 | 1.5 | 0.44 | 0.88 | 0.90 | [a] | 1.6 | 0.34 |
| Triclosan | 4.2 | 2.1 | 2.3 | 2.8 | 2.5 | 2.2 | 2.4 | [a] | [a] |
| Trimethoprim | 0.42 | 0.38 | 0.47 | 0.17 | 0.19 | 0.13 | [a] | 0.13 | 0.10 |
| Venlafaxine | 1.1 | 1.2 | 1.6 | 0.97 | 1.1 | 1.1 | [a] | 1.2 | 0.38 |

17 [a] <MDL (Method detection limit)

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**Table D5 Measured concentrations in speedisk extracts from sampling campaign 3. Speedisks were deployed in triplicates (1-3) at two sampling locations: the harbor of Zeebrugge (HZ) and acoastal sampling location near Ostend (OO_X) and in sextuplicates at the harbor of Ostend (HO). Samples from HO were separated into two groups. A first group (HO.1 – HO.3) was extracted, reconstituted in water and used for spiking of algae growth inhibition experiments. The second group was extracted and extracts were split into two equal fractions, one of which was reconstituted in a methanol-water mix acidified with formic acid and EDTA (HO.4 – HO.6 (MeOH)) and the other fraction was reconstituted in water (HO.4 – HO.6 (H2O)). HO.4 – HO.6 samples were used for method comparison. All concentrations are indicated in µg $L^{-1}$.**

| Compound | HO.1 | HO.2 | HO.3 | HO.4 (H2O) | HO.4 (MeOH) | HO.5 (H2O) | HO.5 (MeOH) | HO.6 (H2O) | HO.6 (MeOH) |
|---|---|---|---|---|---|---|---|---|---|
| Acetamiprid | a | a | a | a | a | a | a | a | a |
| Acyclovir | 1.82 | a | 1.67 | a | a | a | a | a | a |
| Alprazolam | a | 0.094 | 0.086 | 0.11 | a | 0.068 | a | a | a |
| Amantadine | a | 1.9 | 2.8 | 2.1 | 1.7 | 1.7 | 1.4 | 1.4 | 2.3 |
| Amitriptyline | a | a | a | a | 0.12 | a | a | 0.070 | a |
| Atenolol | a | a | a | a | a | a | a | a | a |
| Atrazine | a | a | a | a | a | a | a | a | a |
| Bezafibrate | 5.6 | 2.3 | 5.4 | 1.6 | 1.9 | 1.9 | 1.8 | 1.8 | 1.5 |
| Bisoprolol | a | 4.5 | 0.62 | 3.3 | 5.3 | 5.1 | 5.2 | 4.8 | 3.3 |
| Carbamazepine | a | a | a | a | a | a | a | a | a |
| Chloridazon | a | 1.6 | 1.2 | 1.7 | 1.2 | 1.3 | 1.2 | 1.2 | 1.7 |
| Clarithromycin | 0.072 | 0.32 | 0.15 | 0.29 | 0.41 | 0.41 | 0.67 | 0.64 | 0.25 |
| Clothianidin | a | 0.10 | 0.075 | 0.12 | 0.086 | 0.086 | 0.084 | 0.083 | 0.11 |
| DEET | a | a | a | a | a | a | a | a | a |
| Diclorophenoxyacetic acid | a | a | a | a | a | a | a | a | a |
| Diclofenac | 1.0 | 2.2 | 0.90 | 2.4 | 1.3 | 1.9 | 0.97 | 1.5 | 1.3 |
| Diuron | 0.88 | 0.94 | 0.89 | 1.1 | 1.2 | 1.1 | 1.2 | 1.1 | 1.0 |
| Ethylparaben | a | 0.92 | a | 0.96 | 1.3 | 1.2 | 1.2 | 1.1 | 1.1 |
| Flufenacet | a | 0.28 | 0.52 | 0.30 | 0.21 | a | a | a | 0.32 |
| Flumequine | a | 0.11 | a | a | 0.11 | 0.094 | a | 0.068 | a |
| Imidacloprid | 0.15 | 1.6 | 0.65 | 1.5 | 0.98 | 0.85 | 0.77 | 0.74 | 1.4 |
| Irgarol | a | 0.40 | 0.22 | 0.28 | 0.79 | 0.66 | 0.75 | 0.74 | 0.36 |

| Compound | HO.1 | HO.2 | HO.3 | HO.4 (H2O) | HO.4 (MeOH) | HO.5 (H2O) | HO.5 (MeOH) | HO.6 (H2O) | HO.6 (MeOH) |
|---|---|---|---|---|---|---|---|---|---|
| Isoproturon | a | a | a | a | a | a | a | a | a |
| Ketoprofen | a | a | a | a | a | a | a | a | 0.33 |
| Lamivudine | a | a | a | 0.23 | a | a | a | a | a |
| Levofloxacin | a | 0.18 | a | a | a | a | a | a | a |
| Linuron | a | 2.3 | 0.63 | 2.4 | 1.4 | 1.4 | 1.4 | 1.6 | 2.6 |
| Mecoprop | 1.1 | 0.94 | 1.1 | 0.44 | 0.64 | 0.53 | 0.27 | 0.37 | 0.48 |
| Methylparaben | a | a | a | a | a | a | a | a | a |
| Metolachlor | a | 0.90 | 1.1 | 0.92 | 0.45 | 0.45 | 0.40 | 0.41 | 0.92 |
| Metoprolol | a | a | a | a | a | a | a | a | a |
| Metronidazole | a | a | a | a | a | a | a | a | a |
| Naproxen | a | a | a | a | a | 23 | a | a | a |
| Paracetamol | a | a | a | a | a | a | a | a | a |
| Piperonylbutoxide | a | a | a | a | a | 0.23 | 0.90 | 1.00 | a |
| Pirimicarb | a | a | a | a | a | a | a | a | a |
| Propranolol | a | 0.76 | 0.51 | 0.79 | 0.94 | 0.69 | 0.74 | 0.74 | 0.90 |
| Propylparaben | a | a | a | a | a | a | a | a | a |
| Salbutamol | a | a | a | a | a | a | a | a | a |
| Salicylic acid | a | a | a | a | a | a | a | a | a |
| Simazine | a | a | a | a | a | a | a | a | a |
| Sotalol | 6.1 | 16 | 6.6 | 13 | 9.9 | 9.8 | 8.8 | 8.5 | 14 |
| Sulfamethazine | a | 0.066 | 0.027 | 0.057 | 0.031 | 0.055 | 0.026 | 0.036 | 0.033 |
| Sulfamethoxazole | a | 2.3 | 2.0 | 2.7 | 1.5 | 1.7 | 1.4 | 1.6 | 2.5 |
| Terbuthylazine | a | a | a | a | a | a | a | a | a |
| Thiacloprid | a | a | a | a | a | a | a | a | a |
| Thiamethoxam | a | 1.8 | 0.64 | 1.6 | 1.1 | 1.2 | 0.97 | 1.1 | 1.7 |
| Trimethoprim | a | a | a | a | a | a | a | a | a |
| Venlafaxine | a | 3.4 | 2.0 | 3.4 | 4.7 | 4.7 | 2.0 | 3.7 | 3.4 |

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| Compound | HZ.1 | HZ.2 | HZ.3 | OO_X.1 | OO_X.2 | OO_X.3 |
|---|---|---|---|---|---|---|
| Acetamiprid | a | a | a | a | a | a |
| Acyclovir | a | 3.13 | a | a | a | a |
| Alprazolam | 0.074 | 0.087 | a | 0.067 | a | 0.091 |
| Amantadine | 2.1 | 2.6 | 2.3 | 1.9 | a | a |
| Amitriptyline | a | a | a | a | a | a |
| Atenolol | 2.3 | 1.8 | 3.1 | 1.5 | 2.1 | 2.3 |
| Atrazine | 0.75 | 1.0 | a | 2.0 | a | a |
| Bezafibrate | a | 4.5 | 3.6 | 7.1 | 8.9 | 9.9 |
| Bisoprolol | 3.0 | 0.69 | 2.1 | a | a | a |
| Carbamazepine | 8.2 | 8.3 | a | 9.3 | a | a |
| Chloridazon | 1.6 | 1.0 | 2.3 | 1.5 | a | a |
| Clarithromycin | 0.19 | 0.21 | a | 0.11 | a | a |
| Clothianidin | 0.11 | a | 0.12 | 0.067 | a | 0.078 |
| DEET | 2.1 | 4.5 | a | 3.8 | a | 3.6 |
| Dichlorophenoxyacetic acid | 1.1 | 0.77 | 1.2 | 0.32 | 0.40 | 0.63 |
| Diclofenac | 2.3 | 0.79 | 3.2 | 25 | 20 | 13 |
| Diuron | 0.80 | 0.82 | 1.0 | 0.84 | 0.84 | 0.84 |
| Ethylparaben | a | a | 0.76 | 0.88 | a | a |
| Flufenacet | 0.24 | a | a | 0.50 | 0.76 | 0.75 |
| Flumequine | 0.14 | a | a | a | a | a |
| Imidacloprid | 1.3 | 0.40 | 1.9 | 0.61 | a | 0.44 |
| Irgarol | 0.35 | 0.18 | a | 0.16 | a | |
| Isoproturon | 1.1 | 1.1 | a | 1.1 | a | 0.90 |
| Ketoprofen | a | a | a | a | a | a |
| Lamivudine | a | a | a | a | a | a |
| Levofloxacin | 0.26 | a | a | a | a | a |
| Linuron | 1.8 | 0.63 | a | 1.4 | a | 0.71 |
| Mecoprop | 0.93 | 0.98 | 1.5 | 0.89 | 1.0 | 1.1 |
| Methylparaben | 0.41 | 0.35 | 0.48 | a | a | a |
| Metolachlor | 0.80 | a | a | 1.3 | 1.7 | 1.4 |
| Metoprolol | 1.2 | 0.83 | 1.1 | 0.25 | a | a |
| Metronidazole | a | a | a | a | a | a |
| Naproxen | a | a | a | a | a | a |
| Paracetamol | a | a | a | a | a | a |
| Piperonylbutoxide | a | a | a | a | a | a |
| Pirimicarb | 0.14 | 0.87 | 0.23 | 8.6 | a | a |
| Propranolol | 0.88 | 0.62 | 0.75 | 0.25 | a | 0.08 |
| Propylparaben | 0.23 | 0.34 | 0.30 | 0.30 | 0.34 | 0.42 |
| Salbutamol | a | a | a | a | a | a |
| Salicylic acid | 1.0 | 0.62 | 0.66 | 0.35 | 0.41 | 0.14 |
| Simazine | 0.66 | 0.73 | 0.88 | 1.0 | a | a |
| Sotalol | 10 | 5.7 | 17 | 3.4 | 5.0 | 6.1 |
| Sulfamethazine | 0.024 | 0.026 | 0.10 | a | a | a |

| Compound | HZ.1 | HZ.2 | HZ.3 | OO_X.1 | OO_X.2 | OO_X.3 |
|---|---|---|---|---|---|---|
| Sulfamethoxazole | 2.0 | 1.9 | 3.4 | 1.1 | [a] | [a] |
| Terbuthylazine | 0.97 | 1.4 | [a] | 1.9 | [a] | 0.92 |
| Thiacloprid | 0.45 | 0.24 | 0.93 | 0.20 | [a] | 0.24 |
| Thiamethoxam | 1.4 | 0.62 | 2.3 | 0.63 | [a] | [a] |
| Trimethoprim | 0.37 | 0.38 | 0.62 | 0.28 | [a] | 0.33 |
| Venlafaxine | 2.6 | 1.9 | 2.7 | 1.4 | [a] | [a] |

[a] < MDL (Method detection limit)

**Table D6 Measured concentrations in speedisk $H_2O$ extracts from sampling campaign 5. Speedisks were deployed in triplicates (1-3) at two sampling locations: the harbor of Ostend (HO) and the harbor of Zeebrugge (HZ). All concentrations are indicated in µg $L^{-1}$.**

| Compound | HO1 | HO2 | HO3 | HZ1 | HZ2 | HZ3 |
|---|---|---|---|---|---|---|
| Acyclovir | [a] | 0.23 | 0.47 | 0.33 | [a] | 0.78 |
| Alprazolam | 0.083 | 0.21 | 0.16 | [a] | 0.068 | [a] |
| Amantadine | 1.9 | 2.1 | 1.9 | 3.0 | 2.5 | 3.2 |
| Amitriptyline | 0.083 | [a] | 0.065 | [a] | [a] | [a] |
| Atrazine | 1.4 | 1.7 | 1.6 | [a] | 1.0 | [a] |
| Bisoprolol | 5.4 | 7.8 | 7.0 | 0.86 | 0.77 | 0.83 |
| Carbamazepine | 9.5 | 13 | 11 | [a] | 6.1 | [a] |
| Chloridazon | 3.7 | 5.3 | 4.3 | 1.3 | 1.5 | 1.2 |
| Clarithromycin | 0.12 | 0.43 | 0.44 | [a] | 0.18 | [a] |
| Clothianidin | 0.12 | 0.14 | 0.12 | 0.10 | 0.13 | 0.10 |
| DEET | 2.9 | 3.5 | 3.1 | [a] | 2.2 | [a] |
| Dichlorophenoxyacetic acid | 2.4 | 3.2 | 2.7 | 1.1 | 1.2 | 1.1 |
| Diclofenac | 11 | 15 | 12 | 2.3 | 2.2 | 2.0 |
| Diuron | 1.1 | 1.2 | 1.1 | 0.77 | 0.81 | 0.71 |
| Flufenacet | [a] | 1.7 | 1.5 | [a] | 0.31 | 0.87 |
| Flumequine | 0.16 | 0.19 | 0.16 | [a] | [a] | [a] |
| Imidacloprid | 1.7 | 2.1 | 1.9 | 0.83 | 0.95 | 0.71 |
| Irgarol | 0.070 | 0.083 | 0.078 | [a] | 0.10 | [a] |
| Isoproturon | 1.4 | 1.7 | 1.5 | [a] | 0.82 | [a] |
| Ketoprofen | 1.5 | 1.7 | 1.4 | [a] | [a] | [a] |
| Levofloxacin | 0.23 | 0.21 | [a] | [a] | [a] | [a] |
| Linuron | 0.52 | 0.86 | 0.74 | [a] | [a] | [a] |
| Mecoprop | 1.4 | 1.6 | 1.4 | 1.2 | 1.2 | 1.1 |
| Metolachlor | [a] | 1.5 | 1.4 | [a] | 0.92 | 0.91 |
| Metoprolol | 1.7 | 2.3 | 2.0 | 0.72 | 0.69 | 0.72 |
| Metronidazole | 0.29 | 0.37 | 0.26 | [a] | 0.09 | [a] |
| Naproxen | [a] | 22 | [a] | [a] | [a] | [a] |
| Nevirapine | [a] | [a] | [a] | 0.58 | 0.59 | 0.38 |
| Piperonylbutoxide | 0.94 | 1.4 | 1.4 | [a] | [a] | [a] |
| Pirimicarb | 0.23 | 0.34 | 0.29 | 0.056 | 0.065 | 0.043 |
| Propranolol | 1.7 | 1.7 | 1.8 | 0.51 | 0.30 | 0.81 |

| Compound | HO1 | HO2 | HO3 | HZ1 | HZ2 | HZ3 |
|---|---|---|---|---|---|---|
| Sotalol | [a] | [a] | 30 | 4.9 | 5.7 | 4.0 |
| Sulfadoxin | 0.036 | 0.045 | 0.034 | [a] | [a] | [a] |
| Sulfamethazine | 0.23 | 0.22 | 0.29 | 0.030 | 0.039 | 0.042 |
| Sulfamethoxazole | 3.4 | 4.5 | 3.8 | 1.8 | 1.8 | 1.8 |
| Terbuthylazine | 2.5 | 3.6 | 3.1 | [a] | 0.90 | [a] |
| Thiacloprid | 1.2 | 1.8 | 1.5 | 0.27 | 0.26 | 0.25 |
| Thiamethoxam | 3.3 | 4.8 | 4.3 | 0.98 | 1.1 | 0.93 |
| Trimethoprim | 1.1 | 1.4 | 1.3 | 0.29 | 0.27 | 0.28 |
| Venlafaxine | 3.5 | 4.4 | 4.0 | 1.5 | 1.2 | 1.4 |

[a] < MDL (Method detection limit)

**Table D7 Sample overview for the use in multivariate analysis with PCA and sparse PCA. Sample name includes the sampling campaign (SC), sampling location (HO, HZ, OZ_MOW1 or OO_X), the speedisk replicate number (1-3) and the concentration treatment per speedisk (CT). The significance level indicates statistically significant growth inhibition as compared to control treatments.**

| Sample | Sample name | Growth inhibition [%] | Significance level |
|---|---|---|---|
| 1 | SC2 HO_1 CT1 | 24 | No effect |
| 2 | SC2 HO_2 CT1 | -3.0 | No effect |
| 3 | SC2 HO_3 CT1 | -21 | No effect |
| 4 | SC2 HZ_1 CT1 | 7.6 | No effect |
| 5 | SC2 HZ_2 CT1 | 4.4 | No effect |
| 6 | SC2 HZ_3 CT1 | 8.8 | No effect |
| 7 | SC2 OZ_MOW1 1 CT1 | 43 | Growth inhibition |
| 8 | SC2 OZ_MOW1 1 CT2 | 3.1 | No effect |
| 9 | SC2 OZ_MOW1 2 CT1 | -1.7 | No effect |
| 10 | SC2 OZ_MOW1 2 CT2 | 1.3 | No effect |
| 11 | SC2 OZ_MOW1 3 CT1 | 33 | Growth inhibition |
| 12 | SC2 OZ_MOW1 3 CT2 | 5.7 | No effect |
| 13 | SC3 HO_1 CT1 | 18 | Growth inhibition |
| 14 | SC3 HO_1 CT2 | 15 | No effect |
| 15 | SC3 HO_1 CT3 | 1.1 | No effect |
| 16 | SC3 HO_1 CT4 | -1.7 | No effect |
| 17 | SC3 HO_2 CT1 | 134 | Growth inhibition |
| 18 | SC3 HO_2 CT2 | 24 | Growth inhibition |
| 19 | SC3 HO_2 CT3 | 5.0 | No effect |
| 20 | SC3 HO_2 CT4 | -2.8 | No effect |
| 21 | SC3 HO_3 CT1 | 29 | Growth inhibition |
| 22 | SC3 HO_3 CT2 | 17 | Growth inhibition |
| 23 | SC3 HO_3 CT3 | 2.4 | No effect |
| 24 | SC3 HZ_1 CT1 | 4.2 | No effect |
| 25 | SC3 HZ_1 CT2 | 9.8 | Growth inhibition |
| 26 | SC3 HZ_1 CT3 | -0.57 | No effect |
| 27 | SC3 HZ_2 CT1 | 4.9 | No effect |

| Sample | Sample name | Growth inhibition [%] | Significance level |
|---|---|---|---|
| 28 | SC3 HZ_2 CT2 | 20 | Growth inhibition |
| 29 | SC3 HZ_2 CT3 | -7.2 | No effect |
| 30 | SC3 HZ_3 CT1 | 54 | Growth inhibition |
| 31 | SC3 HZ_3 CT2 | 23 | Growth inhibition |
| 32 | SC3 HZ_3 CT3 | 12 | Growth inhibition |
| 33 | SC3 OO_X 1 CT1 | 214 | Growth inhibition |
| 34 | SC3 OO_X 1 CT2 | 18 | No effect |
| 35 | SC3 OO_X 1 CT3 | 8.6 | No effect |
| 36 | SC3 OO_X.1 CT4 | 0.060 | No effect |
| 37 | SC3 OO_X 2 CT1 | 85 | Growth inhibition |
| 38 | SC3 OO_X 2 CT2 | 22 | Growth inhibition |
| 39 | SC3 OO_X 2 CT3 | -2.9 | No effect |
| 40 | SC3 OO_X 3 CT1 | 162 | Growth inhibition |
| 41 | SC3 OO_X 3 CT2 | 32 | Growth inhibition |
| 42 | SC3 OO_X 3 CT3 | 4.2 | No effect |
| 43 | SC5 HO_1 CT1 | 175 | Growth inhibition |
| 44 | SC5 HO_1 CT2 | 5.4 | No effect |
| 45 | SC5 HO_2 CT1 | 125 | Growth inhibition |
| 46 | SC5 HO_2 CT2 | -4.0 | No effect |
| 47 | SC5 HO_3 CT1 | 96 | Growth inhibition |
| 48 | SC5 HO_3 CT2 | 0.080 | No effect |
| 49 | SC5 HZ_1 CT1 | 138 | Growth inhibition |
| 50 | SC5 HZ_1 CT2 | 8.2 | No effect |
| 51 | SC5 HZ_2 CT1 | 133 | Growth inhibition |
| 52 | SC5 HZ_2 CT2 | 0.11 | No effect |
| 53 | SC5 HZ_3 CT1 | 132 | Growth inhibition |
| 54 | SC5 HZ_3 CT2 | 5.9 | No effect |

**Table D8 Measured pH in algae growth inhibition tests performed with speedisk passive sampling extracts. pH was measured for the lowest and highest concentration treatments at test start (pH0) and test end (pH72). CTL indicates control treatments. Locations: HZ = Harbor Zeebrugge, HO = Harbor Ostend, SZ = coastal sampling location near Zeebrugge, SO = coastal sampling location near Ostend. NA = Not applicable.**

| Sampling campaign (SC) & location | Replicate | Treatment | pH0 | pH72 |
|---|---|---|---|---|
| SC2 CTL | 1 | NA | 7.88 | 8.05 |
| | 2 | NA | 7.89 | 8.08 |
| SC2 HZ | 1 | lowest | 7.88 | 8.03 |
| | 1 | highest | 7.88 | 8.04 |
| | 2 | lowest | 7.87 | 7.91 |
| | 2 | highest | 7.91 | 8.04 |
| | 3 | lowest | 7.85 | 8.03 |
| | 3 | highest | 7.88 | 8.06 |
| SC2 HO | 1 | lowest | 7.90 | 8.04 |
| | 1 | highest | 7.88 | 8.03 |
| | 2 | lowest | 7.86 | 8.00 |
| | 2 | highest | 7.87 | 8.04 |
| | 3 | lowest | 7.87 | 8.02 |
| | 3 | highest | 7.88 | 8.03 |

| Sampling campaign (SC) & location | Replicate | Treatment | pH0 | pH72 |
|---|---|---|---|---|
| SC2 SZ | 1 | lowest | 7.86 | 8.04 |
| | 1 | highest | 7.87 | 8.06 |
| | 2 | lowest | 7.88 | 8.02 |
| | 2 | highest | 7.89 | 8.08 |
| | 3 | lowest | 7.84 | 8.01 |
| | 3 | highest | 7.88 | 8.09 |
| SC3 CTL | 1 | NA | 7.90 | 8.23 |
| | 2 | NA | 7.87 | 8.24 |
| SC3 HZ | 1 | lowest | 7.94 | 8.15 |
| | 1 | highest | 7.93 | 8.16 |
| | 2 | lowest | 7.91 | 8.18 |
| | 2 | highest | 7.90 | 8.15 |
| | 3 | lowest | 7.92 | 8.11 |
| | 3 | highest | 7.92 | 8.06 |
| SC3 HO | 1 | lowest | 7.78 | 8.21 |
| | 1 | highest | 7.73 | 8.18 |
| | 2 | lowest | 7.89 | 8.22 |
| | 2 | highest | 7.86 | 8.19 |
| | 3 | lowest | 7.90 | 8.15 |
| | 3 | highest | 7.93 | 8.12 |
| SC3 SO | 1 | lowest | 7.93 | 8.04 |
| | 1 | highest | 7.93 | 7.97 |
| | 2 | lowest | 7.95 | 8.20 |
| | 2 | highest | 7.91 | 8.18 |
| | 3 | lowest | 7.86 | 8.21 |
| | 3 | highest | 7.86 | 8.20 |
| SC5 CTL | 1 | NA | 7.72 | 8.18 |
| | 2 | NA | 7.72 | 8.20 |
| SC5 HZ | 1 | lowest | 7.66 | 7.96 |
| | 1 | highest | 7.64 | 7.99 |
| | 2 | lowest | 7.70 | 7.99 |
| | 2 | highest | 7.67 | 8.09 |
| | 3 | lowest | 7.72 | 8.08 |
| | 3 | highest | 7.69 | 8.16 |
| SC5 HO | 1 | lowest | 7.65 | 8.11 |
| | 1 | highest | 7.56 | 8.15 |
| | 2 | lowest | 7.66 | 8.17 |
| | 2 | highest | 7.63 | 8.12 |
| | 3 | lowest | 7.67 | 8.11 |
| | 3 | highest | 7.61 | 8.17 |

**Table D9 Loadings of the sparse PCA for the 9 included principal components (PC).**

| Compound | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 | PC9 |
|---|---|---|---|---|---|---|---|---|---|
| Acyclovir | 0 | 0 | 0 | 0 | 0 | -0.020 | 0 | -0.12 | 0.024 |
| Alprazolam | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Amantadine | 0 | 0 | 0 | 0 | 0.0048 | 0 | 0.72 | 0 | 0 |
| Amitriptyline | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Atenolol | -0.21 | 0.10 | 0.54 | -0.0014 | 0.40 | -0.66 | 0.049 | -0.044 | 0.050 |
| Atrazine | 0 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bezafibrate | -0.05 | -0.14 | -0.53 | -0.17 | 0.76 | 0 | 0.00040 | 0 | 0.0097 |
| Bisoprolol | -0.15 | 0.0017 | 0.29 | 0 | 0 | 0 | 0 | 0 | 0 |
| Carbamazepine | -0.48 | 0 | 0 | -0.85 | -0.21 | 0 | 0 | 0 | 0 |
| Chloridazon | -0.13 | 0.014 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

| Compound | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 | PC9 |
|---|---|---|---|---|---|---|---|---|---|
| Clarithromycin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Clothianidin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DEET | -0.0032 | 0 | 0 | -0.033 | 0 | -0.012 | 0 | 0 | 0.65 |
| Dichlorophenoxyacetic acid | 0 | 0 | 0 | 0 | 0 | 0 | 0 | -0.039 | 0.14 |
| Diclofenac | -0.54 | 0.72 | -0.27 | 0.32 | -0.035 | 0 | 0.00086 | 0 | 0 |
| Dimethoate | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Dinoseb | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Diuron | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ethylparaben | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Flufenacet | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Flumequine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Imidacloprid | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Irgarol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Isoproturon | -0.017 | 0.026 | 0 | 0 | -0.061 | 0.12 | 0 | 0.85 | 0 |
| Ketoprofen | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Levofloxacin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Linuron | 0 | 0 | 0 | 0 | -0.011 | 0.016 | 0.10 | 0 | 0 |
| Mecoprop | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Methylparaben | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Metolachlor | 0 | 0 | 0 | 0 | 0 | 0 | 0.0060 | 0.11 | 0.14 |
| Metoprolol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Metronidazole | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Naproxen | 0 | 0.24 | 0.42 | -0.075 | 0.40 | 0.68 | 0 | -0.048 | 0 |
| Nevirapine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Paracetamol | -0.022 | 0.075 | 0.18 | -0.018 | 0.053 | -0.043 | -0.0013 | 0 | 0 |
| Piperonylbutoxide | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pirimicarb | 0 | 0.024 | -0.18 | 0 | -0.24 | 0.24 | 0.13 | -0.37 | 0.14 |
| Propranolol | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Propylparaben | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Salicylic acid | 0 | 0 | 0 | 0.024 | 0 | 0 | 0 | -0.0071 | 0 |
| Simazine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sotalol | -0.63 | -0.63 | 0.067 | 0.38 | 0 | 0.17 | 0 | 0 | 0.00048 |
| Sulfadoxin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sulfamethazine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sulfamethoxazole | 0 | 0 | 0.074 | 0 | 0 | 0 | 0.67 | 0 | 0 |
| Terbuthylazine | 0 | 0 | 0 | 0 | 0 | 0 | -0.0045 | 0 | 0.68 |
| Terbutryn | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thiacloprid | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thiamethoxam | 0 | 0 | 0.13 | 0 | 0 | 0 | 0 | 0 | 0 |
| Triclosan | 0 | 0 | 0 | 0.075 | 0 | 0.062 | 0 | 0.26 | 0.24 |
| Trimethoprim | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Venlafaxine | 0 | 0 | 0 | 0 | 0 | 0 | 0 | -0.18 | 0 |

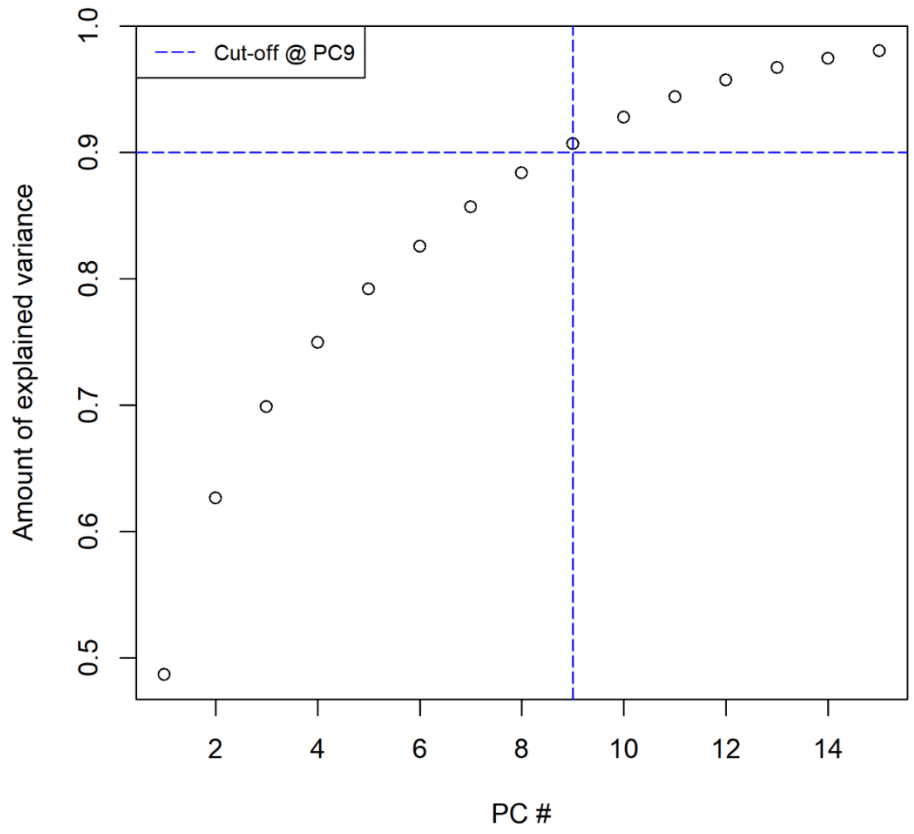


**Figure D1 Cumulative variance plot of the PCA performed for speedisk extracts.**

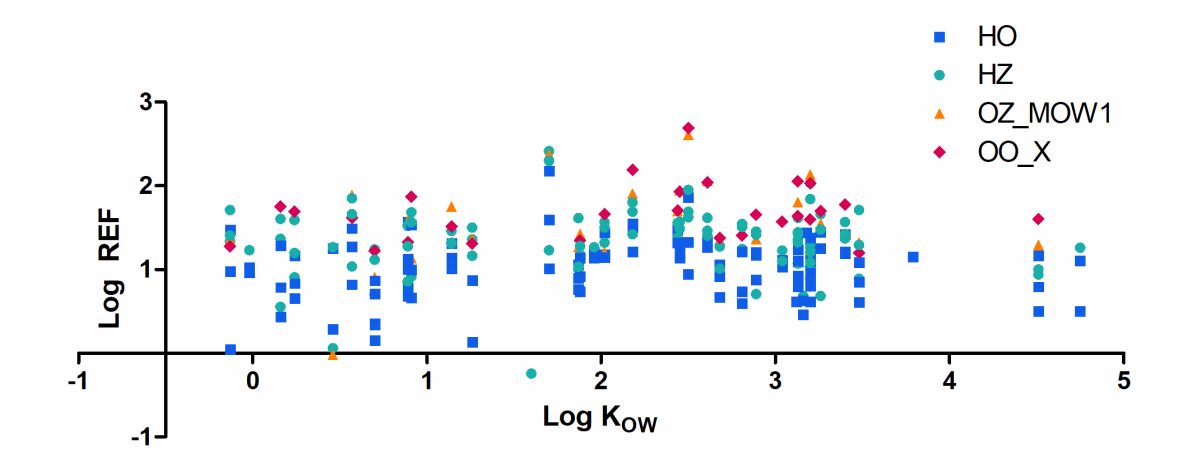


**Figure D2 Correlation plot of the log relative enrichment factor vs the log $K_{OW}$ for all quantified target substances across all sampling locations and campaigns. The sampling locations depicted represent the of harbor of Ostend (HO), harbor Zeebrugge (HZ), coastal sampling location near Zeebrugge (OZ_MOW1) and coastal sampling location near Ostend (OO_X).**

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# Curriculum vitae

## Curriculum vitae

### PERSONALIA


| | |
|---|---|
| Name: | Samuel Moeris |
| Address: | Bergstraße 20<br>4730 Raeren<br>Belgium |
| Date of birth: | 21/05/1988 |
| Place of birth: | Eupen, Belgium |
| Telephone number: | +32 472 62 19 02 |
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### PROFESSIONAL EXPERIENCE

| | |
|---|---|
| 04/2020 – present | Global Product Stewardship Scientist (Global Chemical Management) at Procter & Gamble Services Company NV, Strombeek-Bever, Belgium |
| 10/2015 – present | PhD researcher (Applied Biological Sciences) at Laboratory of Environmental Toxicology and Aquatic Ecology, Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University |

### EDUCATION

| | |
|---|---|
| 10/2015 – present | PhD researcher (Applied Biological Sciences) at Laboratory of Environmental Toxicology and Aquatic Ecology, Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University |
| 10/2012 – 09/2015 | Master of Science in Ecotoxicology at RWTH Aachen University, Germany |
| 10/2008 – 09/2012 | Bachelor of Science in Biology at RWTH Aachen University, Germany |

## OTHER (VOLUNTEERING) EXPERIENCE

| | |
|---|---|
| 02/2019 | Chair of the Young Environmental Scientists (YES) meeting 2019, Ghent, Belgium |
| 05/2017 – present | Member of the Student Advisory Council, Certification Program, Development and Regional Branches Committees of the Society of Environmental Toxicology and Chemistry (SETAC) Europe |
| 09/2013 – 05/2014 | Research internship at the Department of Environmental Science and Analytical Chemistry, Stockholm University, Sweden |
| 09/2004 – 08/2017 | Scout leader (regional and national level) at Les Scouts ASBL |
| 10/2008 – 09/2015 | Logistics and Production Assistant (Job student) at Distillery Radermacher S.A. |
| 04/2011 – 07/2013 | Graduate assistant at RWTH Aachen University |

## ADDITIONAL TRAINING

| | |
|---|---|
| 05/2019 | The use of (Q)SAR for environmental endpoints within the REACH framework at SETAC Europe Annual Meeting, Helsinki, Finland |
| 05/2018 | High-Dimensional Data Analysis at Ghent University, Belgium |
| 02/2018 | Effective professional networking at Ghent University, Belgium |
| 10/2017 | Introduction to R at Ghent University, Belgium |
| 05/2017 | Evaluation of ecotoxicity and degradation studies for use in environmental risk assessment of chemicals at SETAC Europe Annual Meeting, Brussels, Belgium |
| 02/2017 – 03/2017 | Advanced Academic English: Conference skills – Effective slide design & Academic posters at Ghent University, Belgium |

11/2017 Communication skills at Ghent University, Belgium

## PUBLICATIONS

### Journal articles (A1)

- **Moeris, S.**, Vanryckeghem, F., Demeestere, K., Huysman, S., Vanhaecke, L., De Schamphelaere, K.A.C., 2019. Growth stimulation effects of environmentally realistic contaminant mixtures on a marine diatom. *Environmental Toxicology and Chemistry 38(6): 1313-1322.*
- Luederwald, S., Newton, K., Heye, K., Bitter, K., **Moeris, S.**, Benner, L., Boehm, P., Koch, J., Feckler, A., Castro, M., 2018. SETAC GLB and SETAC Europe SAC: a liaison promoting the next generation of ecotoxicologists and environmental chemists. *Environmental Sciences Europe 30.*
- **Moeris, S.**, Vanryckeghem, F., Demeestere, K., De Schamphelaere, K.A.C. Neonicotinoid insecticides from a marine perspective: Acute and chronic copepod testing and derivation of Environmental Quality Standards. *Environmental Toxicology and Chemistry, under review.*
- **Moeris, S.**, Vanryckeghem, F., Demeestere, K., De Schamphelaere, K.A.C. A margin of safety approach for the assessment of environmentally realistic chemical mixtures in the marine environment based on combined passive sampling and ecotoxicity testing. *Science of the total environment, under review.*
- Hansul, S.*, **Moeris, S.***, Huysman, S., Vanryckeghem, F., Demeestere, K., Vanhaecke, L., De Schamphelaere, K.A.C. An automated approach for screening-level risk assessment and prioritization of chemicals of emerging concern. *Environmental Science and Technology, to be submitted.*
  **Shared first authors*

### Conference Contributions

- **Moeris, S.**, Vanryckeghem, F., Huysman, S., Vanhaecke, L., Demeestere, K., De Schamphelaere, K.A.C. Combining chemical and ecotoxicological monitoring of emerging polar micropollutants: towards an integrated environmental risk assessment approach. SETAC North America 40th Annual Meeting, Toronto, Canada, 03 – 07 November 2019. *Platform presentation*.
- **Moeris, S.**, Vanryckeghem, F., Janssen, C.R., Demeestere, K., De Schamphelaere, K.A.C. Environmental risk assessment of emerging organic micropollutants in the Belgian part of the North Sea. SETAC Europe 29th Annual Meeting, Helsinki, Finland, 26 – 30 May 2019. *Poster presentation*.
- **Moeris, S.**, Vanryckeghem, F., Demeestere, K., De Schamphelaere, K.A.C. Severe effects of neonicotinoid insecticides on *Nitocra spinipes* under different exposure conditions. SETAC Europe 29th Annual Meeting, Helsinki, Finland, 26 – 30 May 2019. *Poster presentation*.

- **Moeris, S.**, Koch, J., De Schamphelaere, K.A.C. Testing of realistic contaminant mixtures with the harpacticoid copepod species Nitocra spinipes using passive sampler extracts. SETAC Europe 28th Annual Meeting, Rome, Italy, 13 – 17 May 2018. *Poster presentation*.
- **Moeris, S.**, Vanryckeghem, F., Huysman, S., Demeestere, K., Vanhaecke, L., Van Langenhove, H., Janssen, C.R., De Schamphelaere, K.A.C. Using no observed effects to identify main contributing micropollutants in mixture toxicity assessment. SETAC Europe 28th Annual Meeting, Rome, Italy, 13 – 17 May 2018. *Platform presentation*.
- **Moeris, S.**, Vanryckeghem, F., Huysman, S., Demeestere, K., Vanhaecke, L., Van Langenhove, H., Janssen, C.R., De Schamphelaere, K.A.C. Ecotoxicity testing of environmentally realistic contaminant mixtures using passive samplers : what can we learn from repeating toxicity tests over an extended period of time? VLIZ Marine Science Day, Bredene, Belgium, 21 March 2018. *Platform presentation*.
- **Moeris, S.**, Vanryckeghem, F., Demeestere, K., Van Langenhove, H., Janssen, C.R., De Schamphelaere, K.A.C. Single substance and mixture toxicity of emerging polar micropollutants detected in the marine environment. SETAC Europe 27th Annual Meeting, Brussels, Belgium, 07 – 11 May 2017. *Poster presentation*.
- **Moeris, S.**, Sobek, A., Smith, K.E.C., Seiler, T.B., Breitholtz, M., Hollert, H. Testing single substance and mixture toxicity of antifungal imidazoles with *Daphnia magna* in acute immobilization tests using passive dosing. SETAC Europe 4th Young Environmental Scientists Meeting, Petnica, Serbia, 14 – 19 March 2015. *Platform presentation*.